Regulation of Scaffold Protein PDZK1 and its Impact on the Drug Transporter OATP2B1

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Para os meus pais

Für Sabina

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Abbreviations

ABC	=	ATP binding cassette
ADME	=	Absorption, distribution, metabolism and elimination
BCRP	=	Breast cancer resistance protein
CFTR	=	Cystic fibrosis transmembrane conductance regulator
E_1S	=	Estrone 3-sulfate
ER	=	Estrogen receptor
GWAS	=	Genome wide association studies
HDL	=	High-density lipoprotein
HNF1	=	Hepatocyte nuclear factor 1
HNF4	=	Hepatocyte nuclear factor 4
LXR	=	Liver X receptor
MDR	=	Multiple drug resistance
MRP	=	Multidrug resistance protein
NHERF	=	Na^+/H^+exchanger regulatory factor
OAT	=	Organic anion transporter
OATP	=	Organic anion transporting polypeptide
OCT	=	Organic cation transporter
P-gp	=	P-glycoprotein
PDZ	=	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PDZK1	=	PDZ domain containing 1
PPAR	=	Peroxisome proliferator-activated receptor
PXR	=	Pregnane X receptor
SLC	=	Solute carrier
SLCO	=	Solute carrier organic anion
SNP	=	Single nucleotide polymorphism
SR-BI	=	Scavenger receptor class B type 1
T3	=	Triiodothyronine
T4	=	Thyroxine
THR	=	Thyroid hormone receptor
V_{max}	=	Maximal (transport) velocity

Summary

The PDZ domain containing 1 (PDZK1) is a scaffold and PDZ protein of the Na^+/H^+exchanger regulatory factor (NHERF) family. PDZ proteins consist of one or more PDZ domains that recognizes specific amino acid sequences at the C-terminus of membrane proteins such as transporters or receptors. PDZK1 harbors four PDZ domains that organize multiprotein complexes by protein-protein interaction whereby stabilizing the targeted proteins at the membrane and modulating their function due to the spatial organization of the interaction partners. Transporters influenced by PDZK1 are members of the solute carrier (SLC) or the ATP binding cassette (ABC) superfamily, which are associated to drug metabolism and to the cellular uptake or efflux of endogenous substrates of the thyroid and uric acid homoeostasis.

Genome wide association studies (GWAS) reported an association between single nucleotide polymorphisms (SNP) located in the promoter of scaffold protein and uric acid levels in the tested patients. Interestingly, many of the transporters involved in the handling of the uric acid such as SLC22A12 (URAT1) or ABCC4 (MRP4) were previously shown to directly interact with PDZK1. These data created the hypothesis of a "urate transportosome", a network with PDZK1 orchestrating a multi protein complex of influx and efflux transporters responsible for handling uric acid in the proximal tubulus of the kidney. A central transcriptional factor of the proteins in a "transportosome" maybe a possibility to synchronize a functional unit like the "urate transportosome" to achieve a coordinated secretion and absorption of urate.

In chapter 3.1 we generated further evidence for the "urate transportosome" by reporting a correlation between the expression of urate transporters and of PDZK1 and of the hepatocyte nuclear factor 1 α (HNF1α), a transcription factor. Additionally, HNF1α was shown to regulate PDZK1 expression. First, we observed a transactivation of the promoter of the scaffold protein in presence of HNF1α. Furthermore, the overexpression of the homoebox in primary kidney cell (RPTEC) augmented the mRNA and the protein content of PDZK1. Deletion of the most likely HNF1α binding site on the PDZK1 promoter as well as the ChIP assay verified the direct interaction of the transcription factor with the scaffold protein,

The presence of PDZK1 is not limited to the apical membrane of the proximal tubulus cells in the kidney although it is the tissue with the highest expression. The scaffold protein was also detected in hepatocytes. One of the major focus concerning PDZK1 and its expression at the sinusoidal membrane of the hepatocytes is the involvement in the reverse cholesterol transport by its influence on the Scavenger Receptor B 1 (SR-B1). SR-B1, a High-Density Lipid protein (HDL) receptor, mediates the uptake of cholesterol from HDL particles in liver and other tissues. Nevertheless, the treatment of mice with the TO 901317 an agonist of the central regulator of lipid homoeostasis the liver x receptors (LXRα, NR1H3 and LXRβ, NR1H2), did not affect PDZK1, but reduced the expression of SR-B1. However, TO 901317 does not only bind to LXR but also to the nuclear receptor pregnane x receptor (PXR, NR1I2) well known for its impact in drug metabolism.

In chapter 3.2 we investigated the role of the LXR and PXR in the regulation of PDZK1. LXR increased while PXR reduced the promoter activity of the scaffold protein. Further, the treatment of HepG2 with a specific LXR

agonist (GW3965) increased the mRNA expression. In contrast, the exposition to TO 901317 (LXR and PXR agonist) reduced the protein amount of PDZK1. The data of this chapter presented two new regulators of scaffold protein, LXR a central regulator of the lipid homoeostasis and PXR involved in the protection of the organism against potential harmful xenobiotics.

On the basis of the stabilizing effect of PDZK1 on transporters therefore increasing cellular exchange of compounds one could assume the involvement of modulators of the metabolic rate in the transcriptional regulation of the scaffold protein. In chapter 3.3 the thyroid hormone receptors (THRα, NR1A1 and THRβ, NR1A2), which are essential regulators of the basal metabolic rate, were reported to increase the mRNA and protein expression of PDZK1 after activation by triiodothyronine (T3) in Caco-2 cells. The binding site of THRβ included one of the abovementioned urate homoeostasis associated SNPs namely rs1967017 and the polymorphism impacted the transactivation of the scaffold protein promoter. Additional evidence for the THRβ binding site being located between -4022bp and -4010bp of PDZK1 promoter was obtained by deletion of the binding site and a ChIP-assay.

The change of PDZK1 expression by central regulators of a gene network may indicate that other proteins connected to this system are targeted by the scaffold protein to modulate their function. In this context regarding the regulation of PDZK1 by thyroid hormones, the existence of PDZ binding motif at the C-terminus of the organic anion transporting polypeptide 2B1 (OATP2B1), and the observation by *Leuthold et al.* that thyroxine (T4) is a substrate of this membrane transporter, we investigated the potential modulation of OATP2B1 by the scaffold protein. The data were summarized in chapter 3.4 showing a higher quantity of OATP2B1 at the membrane in presence of PDZK1. The enhanced abundance of the transporter translated in a higher transporter rate (V_{max}) of the canonical substrate (E_1S) of the membrane transporter. In addition, a direct interaction between both proteins was shown using the FRET method. The importance of the PDZ binding motif of OATP2B1 for the interaction with PDZK1 was investigated by comparison of V_{max} between the wild type and OATP2B1 lacking the binding motif. The absence of PDZ binding motif abolished the effect of PDZK1 on OATP2B1.

In chapter 3.5 we focused on the link between OATP2B1 and thyroid hormones. Previously reported data already observed T4 as substrate of OATP2B1 and our data not only supported this finding, but also added T3 to the compounds transported by the membrane transporter. Concerning the regulation of OATP2B1 by thyroid hormones an increase in mRNA as well as protein expression was observed after treating Caco-2 with T3 and T4. Noteworthy is that the regulation is likely to be cell/tissue specific since in Huh7 the presence of T3 and T4 did not affect the mRNA and protein expression of OATP2B1.

1 Introduction

In times of personalized medicine, the understanding of mechanisms contributing to inter-individual differences in gene expression and activity is an integral part of research in pharmacology in order to maximize the efficacy of drugs and minimize side effects. The outcome of a therapy is influenced by changes in regulation and activity of the involved proteins. For example, the acceleration of drug metabolism can lead to intoxication or ineffectiveness, even if the drug was administrated in correct form and dose. Research on membrane transporters, receptors and cytosolic proteins involved in the absorption, distribution, metabolism and elimination (ADME) of xenobiotics can improve therapy or enable it.

A perfect drug must have ideal properties in pharmacodynamics and pharmacokinetics. Pharmacodynamics is the understanding of biochemical and physiological modifications that occur with exposition to a certain compound and this is crucial for a successful therapy. Equally important is the question of what happens with the drug in the organism. The pharmacokinetics relates to the understanding of drug properties concerning ADME. Only a successful interplay of both disciplines guaranties a positive outcome of therapies with xenobiotics, which were discovered or/and synthesizes to improve health or to treat diseases. Disclosing the key proteins impacting on pharmacokinetics or pharmacodynamics of drugs and their variety throughout the population is a necessity, especially concerning the high expectation in personalized medicine.

1.1 Transmembrane transport

The cell membrane also called plasma membrane separates the intracellular compartment from the extracellular space and is considered to act as a barrier. This barrier controls the uptake and the efflux of molecules in a passive or active manner. The lipophilic nature of the plasma membrane primarily allows passive diffusion of small apolar molecules, while large and hydrophilic molecules diffuse slowly through the membrane or not at all. Proteins that facilitate the influx and the efflux of drugs which otherwise would not cross the barrier are present in the plasma membrane.

1.2 Drug transporters

A significant quantity of drug transporters mediating transfer of drugs through the cell membrane are representatives of two major protein families. There are multiple membrane proteins shown to be involved in transmembrane transport. Cellular efflux is mediated by members of the ATP binding cassette (ABC) superfamily, whereas the uptake into the cytosol is mainly accomplished by the solute carrier (SLC) family [1].

1.2.1 ABC transporters

In detail, the family of the ABC transporters consists of 49 encoded genes divided into 7 sub-families (A to G) and are expressed in all major organs and barriers [2]. ABC transporters have a high transport capacity and an energy dependency through hydrolysis of ATP in common. Some members of the ABC transporters as ABCB1 (P-gp, MDR1), ABCC1 (MRP1), and ABCG2 (BCRP) are associated with multi-drug resistance (MDR) due to their capacity to efficiently pumping chemotherapeutics out of the cancerous cells. Further they are believed to cause therapy failure in more than 9 out of 10 metastatic cancer patients [3, 4]. Different approaches were undertaken to solve the problem of chemotherapy resistance: 1. the development of MDR transporter inhibitors with higher specificity, 2. Inactivation of transporters by miRNA/siRNA, 3. development antibodies targeting the efflux transporters, and 4. research on non-MDR substrate chemotherapeutic agents [5]. Nevertheless, the same mechanisms acting negatively in cancer therapies in turn protect the organism against toxins and their metabolites [6]. Aside from the much-discussed role of the ABC transporters in cancer therapy the efflux transporters also impact the pharmacokinetics of non-chemotherapeutics by lowering bioavailability (absorption [7]), prevention of accumulation in the brain (distribution [8]), and by pumping compounds out of the body through the liver (metabolism and elimination [9]) and the kidney (elimination [10]). In the past years many drugs such as antihypertensives, lipid-lowering agents, immunosuppressors and of course anticancer drugs were described as substrates of ABC transporters. Subsequently variations of expression or activity of the efflux transporters have a significant impact on the pharmacokinetics of these drugs [1]. However, the pharmacokinetics of drugs is not only dependent on efflux but also on influx transporters.

1.2.2 SLC transporters

In contrast to the efflux transporters, the SLC transporters are responsible for the uptake of compounds into the cells and are expressed throughout the body. The SLC family with more than 300 members subdivided into 52 families, are not directly dependent on energy but facilitate the influx by passive intake or secondary co-transport [11]. While some of the members of the SLC family have been shown to control the physiologic cellular entry of amino acids [12], glucose [13], trace elements [14], vitamins [15] and neurotransmitter uptake [16], there are also multiple transporters involved in pharmacology. Three SLC transporter families are of special interest nowadays: SLC22, SLC47 and SLCO (SLC21) (summarized in fig. 1 [17]). The SLC22 family summarizes organic cation transporters (OCTs), organic zwitterion/cation transporters (OCTNs) and organic anion transporters (OATs). Substrates of these uptake transporters are antidiabetics (metformin, e.g. OCT1), chemotherapeutics (cisplatin, e.g. OCT2), virostatics (acyclovir, e.g. OAT1) or calcium channel blockers (verapamil, e.g. OCTN1) [18]. The second subfamily (SLC47) consists of two transporters: the multidrug and toxin extrusion 1 (MATE1, SLC47A1) and 2 (MATE2, SLC47A2). MATE1 and MATE2 are pH-dependent transporters and were shown to transport metformin, cisplatin and levofloxacin [19, 20]. The third major investigated subfamily of the SLC transporters are the organic anion transporting polypeptides (OATP) [21, 22].

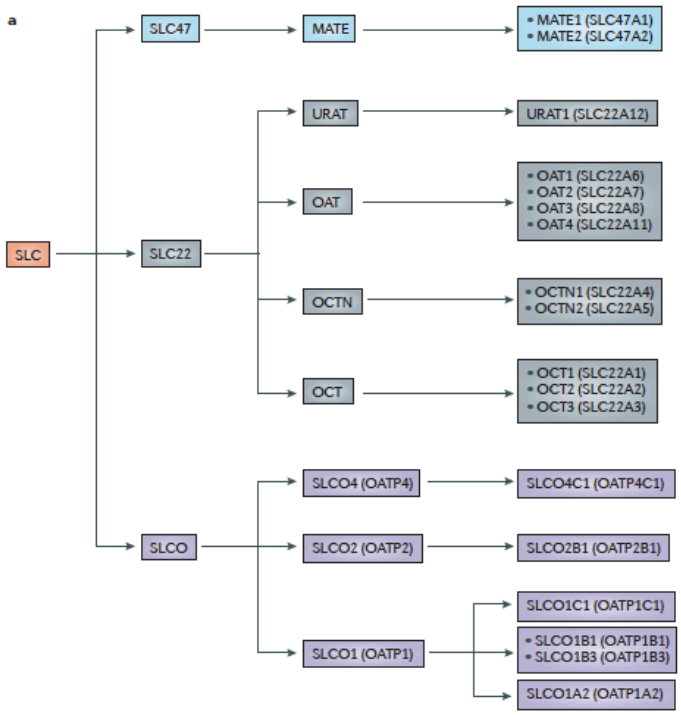

Figure 1: Illustration of the three SLC transporter families involved in drug transport. Overview of the three major SLC families (SLC47, SLC22, and SLCO) focused by research due to their ability to transport drugs (altered figure from [39]).

1.2.3 Organic anion-transporting polypeptides family (OATP, SLC21, SLCO)

In humans the OATPs consist of eleven transporters with twelve transmembrane domains. They facilitate the crossing of cell membranes without being directly dependent on energy from ATP hydrolysis nor the Na^+-gradient [22]. It is assumed that the influxed substrates are the counterparts of effluxed ion molecules such as glutathione resulting in electroneutral exchange [23]. There is evidence for pH sensitivity, which is related to the presence of a highly conserved amino acid in the 3rd transmembrane domain of the OATPs. The only exception is OATP1C1 that harbors at glutamine instead of a histidine [24]. The OATP's are expressed at the membrane of endothelial and epithelial cells of intestine, liver, kidney, testis, blood-brain barrier and placenta (fig. 2 [21]).

Noteworthy is the restriction of some OATPs to specific organs like OATP1B1 in the liver [25-27] or OATP1C1 in the brain and in the testis [28] while other OATPs are expressed throughout the body [21, 29]. Nevertheless, ubiquitously expressed transporters can differ among each other in the distribution e.g. the only OATP present in cholangiocytes is OATP1A2 [30]. In addition, the localization at the membrane can vary as it was described for OATP1A2 and OATP2B1 in the placenta [31, 32]. As the family name suggests the OATPs are primarily transporters of organic anions however they have also been described to transport uncharged, cationic, and zwitterion compounds (summarized in reviews [29, 33]). OATPs are involved in the hormone, bilirubin, and bile acid homoeostasis by transporting compounds and their conjugates relevant to the processes [29]. For instance thyroid hormones are substrates of OATP1A2, -1B1, -1B3, -2B1 -1C1, -3A1, -4A1, -4C1, [24, 25, 28, 34-37]). In more than two decades of

research since the isolation of the first OATP in 1995 (OATP1A2, [38]) a broad range of xenobiotics were described to be transported by OATPs. For example: antibiotics, immune suppressants, beta blockers, HMG-CoA reductase inhibitors (statins), antivirals, ACE-inhibitors, sartans, chemotherapeutics, and antidiabetic agents [29, 33]. The review of *Roth et al.* presents an excellent overview of substrates of the OATPs [29]. However, it should be remembered as pointed out by *Kovacsics et al.* [33] that most experiments were conducted *in vitro* with concentration higher to those detected *in vivo* and therefore the results should be analyzed with caution. A second limitation is the none distinction in several studies whether the compounds are inhibitor or substrate of the examined OATP. Nevertheless, published data in the past years proved that the OATP-family is crucial for the pharmacokinetics of xenobiotics.

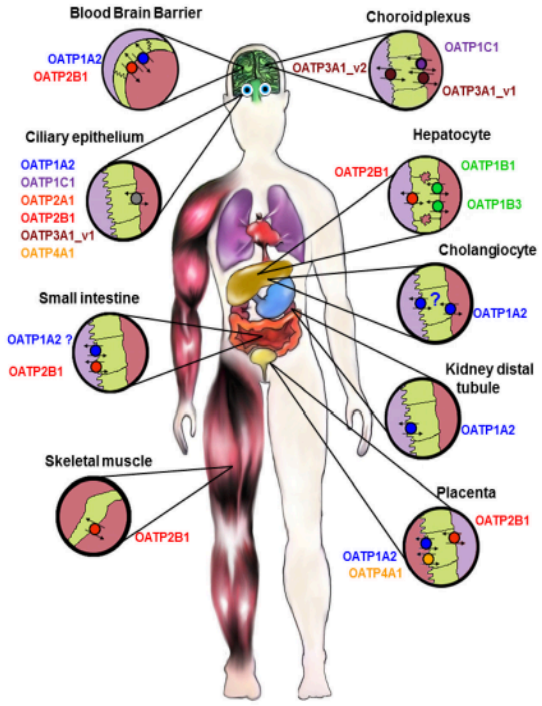

Figure 2: Expression of organic anion transporting polypeptides in the human body. Summary of the appearance of OATPs in various tissues and localization at the apical or basolateral membrane (figure from [43]).

1.2.4 Organic anion-transporting polypeptide 2B1 (SLCO2B1, OATP2B1, OATP-B)

At the turn of this millennium *Tamai et al.* reported on three OATPs with a broad expression profile and the ability to transport estrone 3-sulfate (E_1S) [39], namely OATP2B1, OATP3A1 and OATP4A1. OATP2B1 consists of 709 amino acids and phylogenetic analyses classified it in a new subgroup of the OATPs. In the following years OATP2B1 was shown to be highly expressed in the liver and spleen, but also in various other tissues [39-43]. In detail, OATP2B1 was located at the sinusoidal membrane of the hepatocytes in the liver [40], at the basolateral membrane of syncytiotrophoblasts in the placenta [32], in the vascular endothelium of the heart [42], at the apical membrane of

the intestinal epithelial cells of small intestine [41] and at the apical membrane of endothelial cells of the blood-brain barrier [44]. Further OATP2B1 was located in keratinocytes [45], in the mammary gland [46], in the pars plicata and pars plana of the ciliary body [47, 48], in human platelets [49] and in the skeletal muscle [43]. Consequently, this ubiquitous expression of OATP2B1 suggests that this transporter plays a role in the uptake of numerous drugs and endogenous substrates for many different tissues.

The immense quantity of described endogenous (e.g. prostaglandin E2 (PGE_2, dehydroepiandrosterone-3-sulfate (DEHAS), and thyroxine (T4) [39, 40]), and xenobiotic (bromosulphotphtalein (BSP), statins, antibiotics, anticancer drugs, anti-hypertensive, and fruit juice [39, 41-43, 50]) substrates bears a high risk for drug-drug interaction (substrates of OATP2B1 summarized [29]). Aside from all substrates also inhibitors of the transporters must be taken into account for their interaction potential. *Karlgren et al.* investigated the overlapping and selectivity of inhibitors comparing OATP2B1, OATP1B1 and OATP1B3 [51] all expressed in the main metabolic organ - the liver. They reported specific inhibitors for OATP2B1 such as astemizole, erlotinib, flutamide, itroconazol, levothyroxine, tetracycline and valproic acid [51]. The drug erlotinib used against lung and pancreas cancer showed to be a highly potent and selective inhibitor of OATP2B1 (over 90% inhibition in HEK293 and liver tissue) compared to OATP1B1 and OATP1B3 [51]. Besides the drug inhibitors, citrus juice was shown to affect the OATP2B1 transport *in vitro.* This would have an impact on the intestinal absorption of OAPT2B1 substrates regarding unclear to the expression of the membrane transporter at the membrane of enterocytes [52-54]. In an earlier study from 2007, when patients were given glibenclamide (substrate of OATP2B1) no change in the plasma concentration of the anti-diabetic drug after ingestion of 200 ml of grapefruit juice was reported [52, 55]. In contrast more recent studies looked into the impact of fruit juice on plasma concentration of drugs such as aliskiren [56, 57], celiprolol [58], and fexofenadine [59, 60] investigating the drug interaction with OATPs in the intestine (review by Yu et al [61]). The data of the clinical studies revealed a reduction of the drug absorption by co-administration of certain fruit juices (apple, orange, grapefruit) and suggested the inhibition of the OATP2B1 due to previously reported *in vitro* studies [58, 59, 62-64]. Although most of the above-mentioned drugs are substrates of OATP2B1 they were also transported by OATP1A2 [65-67] and by P-gp which limits the association between the reduced plasma level and the inhibition of OATP2B1. Nevertheless, the observed reduction seems more likely to be dependent on the inhibition of OATP2B1 reasoned by the controversial discussion about the presence of OATP1A2 in the intestine [68-71].

1.3 Factors influencing the function of drug transporters

The information of genes is encoded in certain areas of the DNA which is transcripted to mRNA. The mRNA then serves as template for the translation to proteins. The promoter of a gene is located upstream (5′) of the transcriptional start. A large class of transcriptional factors, the nuclear receptors, are able to bind after activation to specific regions (response elements) on the promoter which finally regulates the transcriptional activity. The transcriptional regulation of every gene contributes to a major part of the later quantity and therefore activity of the protein. Genetic differences located on the promoter or in the translated region of the gene can change the transcription and/or the activity of the protein. Finally, the proteins can also be posttranslational modulated by interaction with other

proteins. The posttranslational modulation referrers to an interference of other proteins after the biosynthesis of the target protein. All these regulations effect many if not all transcribed and translated gene products including proteins of the drug metabolism. Understanding the key factors influencing activity of drug transporters is mandatory to comprehend the variation in the pharmacokinetics of their substrates.

1.3.1 Transcription regulation of OATP2B1

Maeda et al. characterized in 2006 the promoter of OATP2B1 finding several binding sites for transcriptional factors [72]. Performing reporter gene assays with 5' deletion fragments of the promotor they reported that transcriptional activity of OATP2B1 is dependent on SP1 and not on the transcriptional factor HNF1α [72]. Further the transcriptional regulation of OATP2B1 was described to be complex due to isoforms with different transcription start sites [73, 74]. In detail, in the intestine the full-length isoform OATP2B1-1b (consisting of 709 amino acids) is the predominant form compared to the shorter OATP2B1 isoforms (687 amino acids, 1d and 1e) whereas in the liver the isoform 1e demonstrates the highest expression. Interestingly, the isoforms 1b and 1e are differently transactivated. While OATP2B1-1e isoform has been shown to be transactivated by HNF4α, the full-length isoform 1b was not. This suggests a tissue specific regulation of the isoforms [73, 74]. Nonetheless, the regulation of the membrane transporter and its isoform is poorly understood.

1.3.2 Posttranslational modulation by PDZ proteins

Drug transporters are located at certain sites of the cell (fig. 3 [1]). Polarized epithelial and endothelial cells have an apical site directed to the lumen and a basolateral site that is not connected to the lumen. This architecture of the cells in apical and basolateral sites with different protein compositions at the membrane creates the possibility of directed transport of compounds. In a broader pharmacological context directed transport is important for the absorption from the intestine, distribution throughout the blood stream, metabolism in the liver and elimination from the organism in the kidney. As abovementioned drug transporters facilitate the transfer of endogenous and xenobiotic compounds through cellular barriers. Accordingly, the sorting of transporters to a certain cell membrane directed to the apical or basolateral site is essential for the functionality of a barrier. Several members of the ABC- and SLC superfamilies possess a specific amino acid sequence (PDZ ligands or PDZ binding motifs) that can be recognized by PSD-95/Drosophila discs large/ZO-1 (PDZ) domains which is an integral part of the so called PDZ-proteins [75, 76]. These PDZ proteins such as the Na^+/H^+ exchanger regulatory factor (NHERF) family have no direct catalytic role, but function as so-called scaffold protein. Scaffold proteins are involved in the arrangement of multi protein complexes, whereby stabilizing proteins at the membrane, sorting proteins to a specific region of the membrane, facilitating the signal transduction, and/or stimulating the transport activity [77, 78]. The PDZ domains in the PDZ proteins interact with the mainly C-terminally located PDZ binding motif of the target proteins [79-81]. These PDZ binding motifs are divided into three classes determined by the amino acid sequence. Class 1 PDZ binding motifs have the sequence X-[S/T]-X-φ where X can be any amino acid, S/T serine or threonine, and φ is a hydrophobic amino acid. The class 2

PDZ binding motif is characterized by X-φ-X-φ sequence and class 3 PDZ binding motif possesses the sequence -[D/E]-[K/R]-X-φ (D aspartate, E glutamate, K lysine, R arginine). Although there are reports of xenobiotic transporters harboring a class 2 or 3 PDZ binding motif [76, 82, 83] the major part of drug transporters possess a class I PDZ binding motif such as OATP1A2[84].

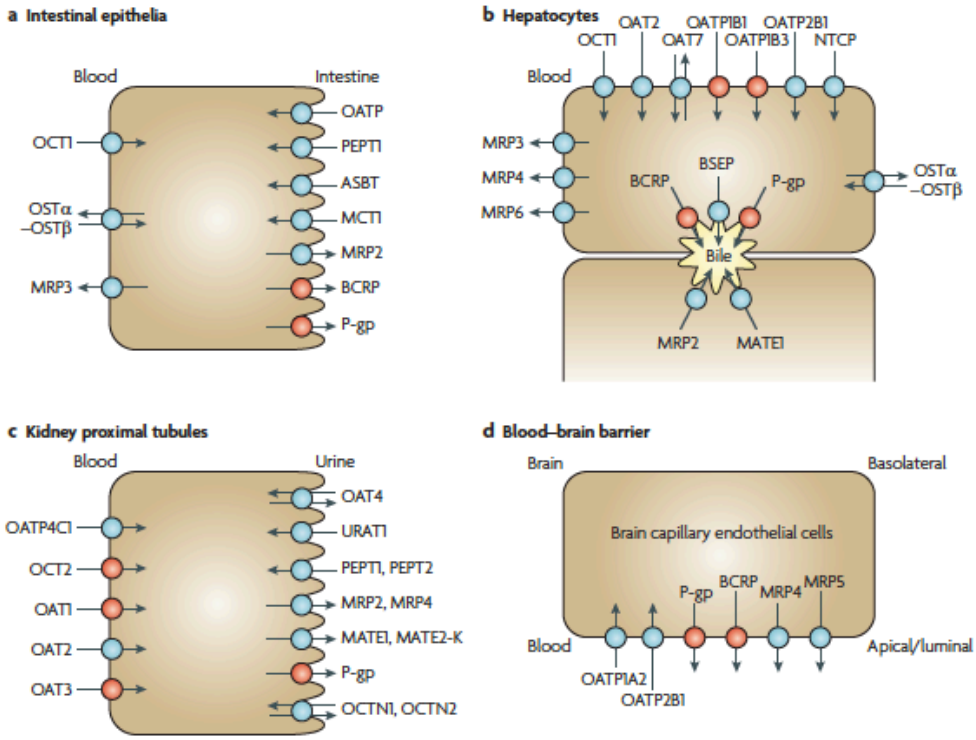

Figure 3: Overview of localization of transporters in different tissues. Illustration of drug transporters in the intestine (a), liver (b), kidney (c), and brain (d). In the intestinal epithelia(a), in hepatocytes (b), and in the kidney proximal tubulus cells (c) the apical membrane is directed to the intestine, bile duct, and the proximal tubulus, respectively while the basolateral membrane always faces the blood. However, in the blood-brain barrier (d) the apical membrane is directed to blood stream while the basolateral membrane oriented to the brain (figure from [23]).

The NHERF family consists of four members all possessing PDZ domains. NHERF1 and NHERF2 carry two PDZ domains and additionally an ezrin-binding domain (EBD) that interacts with the cytoskeleton, while PDZK1 (NHERF3) and NHERF4 (PDZK2) have four PDZ domains, but no EBD (fig. 4). The expression of the four NHERF isoforms differs depending on the tissue and the localization to the apical or basolateral membrane of the cell (summarized by the review of *Walsh et al.*) [75]. Although all NHERFs harbor PDZ domains the affinity to target proteins and spectrum of interacting membrane proteins vary between the scaffold protein isoforms [85-88]. These studies showed the complexity in the formation of multi protein complexes by the NHERF family. The creation of tissue-dependent unique networks is the result of the NHERF family's diverse expression and target protein affinity. One of these networks was described in the kidney for the handling of uric acid and the scaffold protein involved in this multi protein complex was PDZK1 [89].

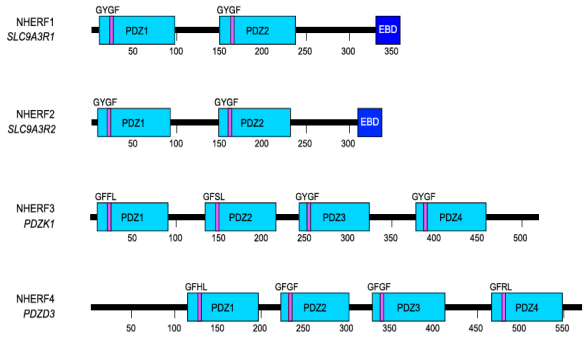

Figure 4: Illustration of the four NHERF family members. In the PDZ domains (light blue) colored purple are the core binding motif with the amino acid sequence above. For NHERF1 and NERHF2 are the ezrin-binding domain (EBD) indicated with a dark blue color (figure from).

1.3.3 PDZK1 (NHERF3, CAP70, CLAMP, DIPHOR-1)

In 1997 *Custer et al.* isolated a new protein with the highest expression in proximal tubulus of the kidney but also in the small intestine of rodents with a molecular weight 52kDa named diphor-1 (dietary Pi-regulated RNA-1) at that time [90]. Diphor-1 increased the Na-P_i cotransport in oocytes and seemed to be related to NHERF1. One year later the group of *Kocher et al*. reported an interaction partner of the MAP17 using a yeast two-hybrid system. The 519 amino acid large protein possessed PDZ domains and was named PDZ containing protein K1 (PDZK1) [91]. PDZK1 was detected in kidney, pancreas, liver, the gastrointestinal tract, and the adrenal cortex and was co-localized with MAP17 in the apical membrane of the proximal tubulus in the kidney [91]. Finally, in the year 2000 a protein with four PDZ domains named CAP70 (CFTR-Associated Proteins kDA 70) was identified interacting with the C-terminus of cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) [92]. Already at the beginning the four PDZ domain containing scaffold protein PDZK1 (although named differently) showed broad spectrum of interactions with proteins of different physiological areas. NaPi-IIa (SLC34A1) is linked to the phosphate

homoeostasis [93], MAP17 (PDZK1 interacting protein 1, PDZK1IP1) a cargo protein correlating with immune response in cancer and inflammatory disease [94] and ABCC7, a chloride channel, where mutations are related to cystic fibrosis [95].

The first analysis of the PDZK1 promotor lead to the discovery of the first transcriptional regulator of the scaffold protein the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor involved in the lipid homoeostasis [96]. PDZK1 was reported to be regulated by estrogen suggesting the involvement of estrogen receptors (ER) in the transcriptional regulation. The scaffold protein was upregulated in ER positive breast cancer [97] and in melisma patients with hyperpigmentation, which is associated with increased estrogen levels [98]. However, the ER did not control the transcription of PDZK1 directly[99]. A target protein of ER the insulin-like growth factor 1 receptor (IGF-1) was shown to directly regulate the transcription of the scaffold protein [99]. Nevertheless, despite the role of the PDZ protein PDZK1 in modulating membrane transporters and receptors the transcriptional regulation of the scaffold protein is largely unknown.

Even though it would be interesting to give a complete insight in all areas in which PDZK1 is involved, this would go beyond the scope of this introduction. Therefore, the following section will mainly focus on interactions with drug transporters such as ABCC2 or OATP1A2.

1.3.4 Impact on drug transporters

One of the first drug transporters described to interact with PDZK1 was ABCC2 (MRP2) [100]. However, the same research group investigated later the effect of the interaction in Pdzk1$^{-/-}$ mice showing no difference in the localization or expression of Abcc2 in proximal tubulus cells suggesting that the scaffold protein is not involved in sorting of the efflux transporter [101]. Nevertheless, the authors also hypothesized about the possibility that the potential effect on MRP2 by the absence of PDZK1 may be masked by functional compensation through other proteins. But still the question remains whether PDZK1 plays a role in the stabilization or localization of MRP2 in liver or intestine.

In contrast the interaction of ABCC4 (MRP4) and PDZK1 is supported by *in vitro* and *in vivo* data. PDZK1 binding to the C-terminal PDZ binding motif of ABBC4 stabilizes the transporter at membranes of HEK293 cells and proximal tubulus cells in kidney, resulting in an increased efflux of the antiviral agent adevofir [86]. In addition, *Park et al*. proofed that the PDZ domain 1 of PDZK1 and the C-terminus of ABCC4 are both essential for the interaction. Further, the data indicated that the underlying mechanism is stabilization of ABCC4 at the membrane and the prevention of internalization by the scaffold protein [86]. The *in vivo* data was generated by conducting experiments investigating the pharmacokinetics of adevofir in Pdzk1$^{-/-}$ mice demonstrating a reduced efflux resulting in an increased AUC and tissue concentration of the antiviral drug [86]. This was explained by the reduced apical presence of Mrp4 in Pdzk1$^{-/-}$ mice and supported by immunohistochemical methods [86].

Noteworthy at this point is the interaction of Pdzk1 with Abcg2 (Bcrp) shown in the small intestine of mice. The scaffold protein plays an important role for the apical localization in enterocytes [83]. This discovery was rather surprising as neither the murine nor the human orthologue of ABCG2 exhibits a canonical PDZ binding motif. The

direct physical interaction between the PDZK1 and BCRP eliminated the possibility of indirect relation. PDZK1 not only interacts with ABC transporters, but also with members of the SLC family. First evidence for an interaction between PDZK1 and several members of the SLC family was provided by *Kato et al.* [76]. The screening of C-terminal sequences of influx transporters for PDZ binding motif disclosed PEPT2, OCT3, OCTN1, OCTN2, OAT4, OATP1A2, OATP1C1, OATP2B1, OATP3A1 and OATP4A1 as potential interaction partners of the NHERF proteins [76]. By using the yeast two-hybrid method with recombinant C-terminals of the transporters they only evaluated some of the abovementioned drug transporters likely to be modulated by PDZK1, namely PETP2, OCTN1, OCTN2, OAT4 and OATP1A2 [76]. In the following years the evidence for a modulation by the scaffold protein increased by studies describing an elevated transport rate and stabilization of transporters at the membrane *in vitro* (PEPT2, OCTN2, OAT4, OATP1A2) and *in vivo* (Octn2) [82, 84, 102-104]. Nevertheless, other SLC transporters not detected as potential partners by *Kato et al.* or not investigated were also shown to be interacting with PDZK1 as SLC22A11 (URAT1), Oatp1a1 or Pept1 [82, 105-107]. The impact of PDZK1 on drug transporters of the SLC and ABC transporters family is mainly a stabilizing effect provoking an abundance that leads to a higher transporter rate[78]. However, the presence of OCTN2 at the membrane did not increase although the transport rate increased by six fold pointing to an ability of NHERF family to influence the activity without increasing the cell surface expression [78, 102].

Interestingly, the aforementioned concept of a “urate transportosome” is a interaction of several membrane transporters with PDZK1 all related to the urate transport at the apical membrane of the renal tubular cells [89]. *Anzai et al* suggested that the handling of uric acid should be assessed by the multi protein complex with SLC (e.g. SLC22A11) and ABC (e.g. ABCC4) transporters modulated by PDZK1 rather than by single transporter analysis [89]. Whether the concept of “transportosomes” can be extended to other tissues (e.g. in the intestinal) and to what dimensions it impacts the drug handling (drug “transportosome”) will be clarified in future studies.

2 Aims of this thesis

The **first aim** of this thesis was to study the transcriptional regulation of PDZK1 by investigating different transcriptional factors as potential regulators of the scaffold protein. The aforementioned "urate transportosome" is a functional unit responsible for the handling of uric acid in the kidney and PDZK1 was shown to posttranslational modulate membrane transporter in this multi protein complex [89]. In addition, a transcriptional factor called hepatocyte nuclear factor 1 homeobox α (HNF1α) had been previously described to regulate the gene expression of several membrane transporter of this "urate transportosome"[108]. In chapter 3.1 we investigated the potential involvement of the HNF1α also as transcriptional regulator of PDZK1.

The scaffold protein is not only interacting with membrane transporter facilitating the uptake or elimination of uric acid but also with receptors of the lipid metabolism [109]. PDZK1 was reported to interplay with the HDL-receptor SR-BI (SCARB1) thereby preventing the membrane protein from degradation [110]. The liver X receptors, LXR α (NR1H3) and LXR β (NR1H2) are nuclear receptors known for their regulation of cholesterol homoeostasis upon activation [111]. Nevertheless, the exposition of mice to the LXR-agonist TO 901317 did not changed the protein expression of the scaffold protein contradicting LXR as regulator of PDZK1 [112]. However, TO 901317 is also a ligand of the nuclear receptor pregnane X receptor (PXR, NR1I2) [113] and in chapter 3.2 we analyzed the role of both nuclear receptors in the regulation of the PDZK1.

At the beginning of chapter 3.3 we analyzed the PDZK1 promoter regarding the single nucleotide polymorphisms (SNP) related associated to certain uric acid plasma levels phenotypes. Interestingly, the individuals harboring a minor allele of the SNP had a trend to a lower mRNA expression of the scaffold protein. To investigate the underlying mechanisms, we screened the promoter with different nuclear receptors discovering thyroid hormone receptor α and b (THRα, NR1A1 and THRβ, NR1A2) as regulators of PDZK1.

The **second aim** of the thesis was to investigate the potential interaction of PDZK1 with OATP2B1 and to characterize the impact of the scaffold protein on the drug transporter. OATP2B1 possesses at the C-terminus a class 1 PDZ binding domain and therefore an interplay between the membrane transporter and PDZK1 is likely and the data was summarized in the chapter 3.4.

In the last chapter 3.5 we focused on the role of OATP2B1 as uptake transporter for thyroid hormones in the intestine as it had been reported that thyroxine [24] is substrate and this membrane transporter is assumed to play an important role in the absorption of the intestine [41].

List of Publications

This doctoral thesis is based on four publications and one manuscript:

Publications

1. Prestin, K., J. Hussner, C. Ferreira, I. Seibert, V. Breitung, U. Zimmermann, and H. E. Meyer Zu Schwabedissen. "Regulation of Pdz Domain-Containing 1 (Pdzk1) Expression by Hepatocyte Nuclear Factor-1alpha (Hnf1alpha) in Human Kidney." *Am J Physiol Renal Physiol* 313, no. 4 (Oct 1 2017): F973-F83.

2. Ferreira, C., K. Prestin, J. Hussner, U. Zimmermann, and H. E. Meyer Zu Schwabedissen. "Pdz Domain Containing Protein 1 (Pdzk1), a Modulator of Membrane Proteins, Is Regulated by the Nuclear Receptor Thrbeta." *Mol Cell Endocrinol* 461 (Feb 5 2018): 215-25.

3. Meyer Zu Schwabedissen, H. E., C. Ferreira, M. A. Schäfer, O. Mouhssin, I. Seibert, M. Hamburger, and R. G. Tirona. ”Thyroid hormones are transport substrates and transcriptional regulators of Organic Anion Transporting Polypeptide 2B1”. Mol Pharmacol, 2018.

4. Ferreira, C., P. Hagen, M. Stern, J. Hussner, U. Zimmermann, M. Grube, and H. E. Meyer Zu Schwabedissen. " The scaffold protein PDZK1 modulates expression and function of the organic anion transporting polypeptide 2B1”. Eur J Pharm Sci, 2018. 120: p. 181-190.

Manuscript

5. Ferreira, C., R. Meyer, and H. E. Meyer Zu Schwabedissen. " The nuclear receptors PXR and LXR are regulators of the scaffold protein PDZK1".

3 Results

3.1 Regulation of PDZ domain-containing 1 (PDZK1) expression by hepatocyte nuclear factor-1α (HNF1α) in human kidney

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Co-author Celio Ferreira contribution: Acquisition, analysis and interpretation of data regarding the deletion of HNF1α binding site on the PDZK1 promoter.

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RESEARCH ARTICLE

Regulation of PDZ domain-containing 1 (PDZK1) expression by hepatocyte nuclear factor-1α (HNF1α) in human kidney

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Prestin K, Hussner J, Ferreira C, Seibert I, Breitung V, Zimmermann U, Meyer zu Schwabedissen HE. Regulation of PDZ domain-containing 1 (PDZK1) expression by hepatocyte nuclear factor-1α (HNF1α) in human kidney. *Am J Physiol Renal Physiol* 313: F973–F983, 2017. First published July 19, 2017; doi:10.1152/ajprenal.00650.2016.—In the renal proximal tubule the secretion and reabsorption of glomerularly filtrated compounds is realized by a functional network of uptake and efflux transporters. The activity and localization of several transporters expressed at the apical tubular membrane are regulated by the membrane-associated protein PDZ domain-containing 1 (PDZK1). We aimed to characterize the transcriptional regulation of this modulator of renal transport. Coexpression analyses of PDZK1 and putative regulators were performed using human kidney samples. Protein and mRNA expression of PDZK1 in renal proximal tubule epithelial cells after adenoviral transfer and siRNA knockdown of transcription factor hepatocyte nuclear factor-1α (HNF1α) was assessed by quantitative real-time PCR and Western blot analysis. Transactivation of the PDZK1 promoter was quantified in cell-based reporter gene assays. Subsequently, the binding of HNF1α to the PDZK1 promoter was verified by in silico analyses and chromatin immunoprecipitation assay. HNF1α positively regulated the promoter activity of PDZK1. Adenoviral overexpression of HNF1α in renal proximal tubule epithelial cells (RPTEC) increased PDZK1 mRNA and protein expression, whereas siRNA knockdown of HNF1α resulted in decreased expression of PDZK1. Our results show that HNF1α, which has previously been described as a modulator of several transporters of the renal transportosome, is also a key determinant of PDZK1 transcription.

uric acid; renal proximal tubule; transport; PDZK1 protein promoter; transcription factor

IN HUMAN RENAL PROXIMAL tubule cells the coordinated function of uptake and efflux transporters determines renal elimination and tubular reabsorption of exogenous and endogenous substances. One example for the functional synergism of membrane transporters is the renal handling of uric acid (28), the final product of purine metabolism. Renal elimination of this hydrophilic compound is based on glomerular filtration, which is limited by tubular reabsorption. Additionally, uric acid is actively secreted in the proximal tubule. It is assumed that urate reabsorption is realized by the function of multiple solute carrier (SLC) transporters, including urate transporter 1 (URAT1, *SLC22A12*), organic anion transporter (OAT) 4 (*SLC22A11*), and OAT10 (*SLC22A13*). These transporters are expressed at the apical membrane, thereby mediating the uptake of their substrates into tubular cells. The glucose transporter -9 (GLUT9, *SLC2A9*), which facilitates urate efflux, is localized at the basolateral membrane of the cell and eliminates reabsorbed urate molecules from the cell into circulation. In contrast, organic anion transporters, such as OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*), localized at the basolateral pole of tubular epithelial cells, govern urate uptake from the blood in the cells, whereas Na^+-dependent phosphate cotransporters NPT1 (*SLC17A1*) and NPT4 (*SLC17A3*) and the ATP-binding cassette (ABC) transporter multidrug resistance-associated protein 4 (*ABCC4*) facilitate the cellular extrusion at the apical membrane (Fig. 1). The concept of a coordinated urate transporter network has previously been introduced as the "urate transportosome" (39).

The importance of transporters in uric acid homeostasis is supported by genomewide association studies identifying genetic variations in genes of this functional network as predictors for serum uric acid levels and/or gout (17). Another factor found to be associated with uric acid homeostasis is the PDZ domain-containing 1 protein PDZK1 (20). This membrane-associated protein harbors four PDZ domains that interact with carboxy-terminal peptide sequences of specific membrane proteins, thereby influencing their subcellular localization and function (12, 15). PDZK1 may be viewed as a scaffold that stabilizes the colocalization of functionally connected proteins. Focusing on the above-mentioned urate transportosome in the kidney, it seems noteworthy that PDZK1 is highly expressed at the apical membrane of tubular epithelial cells and that most of the above-mentioned apical transporters have been reported to directly interact with PDZK1 (1, 2, 9, 21, 23, 32). Indeed, the transport activity of the efflux transporter multidrug resistance-associated protein 4 (MRP4) and the organic anion uptake transporters URAT1 and OAT4 is enhanced in the presence of the scaffolding protein PDZK1 (2, 21, 23). However, although various protein-protein interactions of PDZK1 with numerous membrane transporters have been reported, little is known about the regulation of this putative network. Moreover, elucidating the mechanisms involved in modulation of renal transport is not only a prerequisite for identifying factors regulating serum uric acid levels but also for prediction of pharmacokinetics of drugs that are substrates of the above-mentioned transporters (8).

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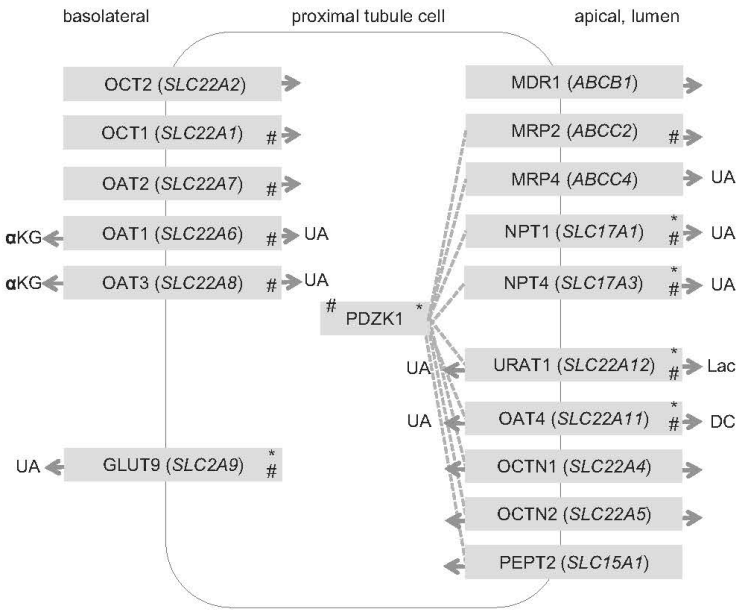

Fig. 1. Renal transporter network and interacting partners of scaffold protein PDZ domain-containing 1 (PDZK1). Shown are transporters expressed in renal proximal tubule cells and transporters of the urate transportosome, membrane transporters transcriptionally regulated by hepatocyte nuclear factor (HNF)-1α and/or HNF4α (#), and transporters harboring genetic variants that are associated with serum urate levels and/or susceptibility to gout (*). Arrows indicate the directions of transport. Broken lines indicate interactions with PDZK1. UA, uric acid; αKG, α-ketoglutarate; DC, dicarboxylate; Lac, lactate; SLC, solute carrier; OCT, organic cation transporter; OAT, organic anion transporter; GLUT, glucose transporter; ABC, ATP-binding cassette transporter; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NPT, Na^+-dependent phosphate transport protein; OCTN, organic cation/carnitine transporter; PEPT, peptide transporter.

Because transcription factors can be considered as crucial modulators of functional gene networks, one may speculate that the genes of the urate transportosome are also coordinately regulated by one factor, thus forming a transcriptional network that provides a synchronized regulation of renal tubule function. Transcription factors previously reported to play a critical role not only in renal developmental but also in the regulation of drug transporters and metabolizing enzymes are the hepatocyte nuclear factor-4α (HNF4α/*NR2A1*) and hepatocyte nuclear factor-1α (HNF1α) (18). Although the nomenclature of these factors suggests that they are exclusively expressed in liver, HNF1α and HNF4α have also been detected in extrahepatic cells (6, 10, 19). HNF4α belongs to the transcription factor family of nuclear receptors and has been reported to modulate expression of OAT1, Oat3, NPT1, NPT4, and GLUT9 (7, 22, 27, 30, 36). Similarly, the homeobox domain factor HNF1α not only regulates liver-specific genes, but also the expression of urate transporters such as Npt1, NPT4, OAT1, OAT3, OAT4, and URAT1 (5, 11, 13, 14, 16, 29, 31).

However, even though there is rising evidence that transcription factors are key determinants of the network summarizing the aforementioned renal transporters, it has not been clarified yet whether PDZK1 as a posttranslational modulator of membrane transporters is also part of the assumed transcriptional gene network that coordinates the expression of drug transporters in the human kidney. Hence, the aim of our study was to characterize the transcriptional regulation of PDZK1.

MATERIALS AND METHODS

Tissue samples. Human kidney tissue samples were obtained from patients undergoing surgery for renal carcinoma. Collection of malignant transformed and adjacent nonmalignant transformed tissue samples was performed after written informed consent. The study was approved by the local ethics committee of the Medical Faculty of the University of Greifswald (III UV 12/03). Nonmalignant transformed tissue samples were prepared as described previously (26).

Cell culture. HeLa cells (CCL-2; ATCC) were cultured in DMEM supplemented with GlutaMAX-I, 1 mM sodium pyruvate, and 10% FCS (Thermo Scientific, Reinach, Switzerland) at 37°C in a humidified 5% CO_2 atmosphere. Human primary renal proximal tubule epithelial cells (RPTEC, Lonza CC-2553, lot 0000324297; RUWAG Lifesciences, Bettlach, Switzerland) were isolated from kidney tissue of a 55-yr-old male and were cultured in basal renal epithelial growth medium 2 supplemented according to the manufacturer's recommendations (Clonetics REGM2; Lonza, Basel, Switzerland). The cells have been proven by the manufacturer to be positive for the expression of the renal epithelial brush-border marker γ-glutamyltransferase 1.

RNA extraction and quantitative real-time RT-PCR. NucleoSpin silica-membrane technology was used to isolate total RNA from surgical tissue and cells, following the manufacturer's protocol (Macherey-Nagel, Dueren, Germany). For reverse transcription of mRNA, the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was applied using a total amount of 2 μg mRNA. Quantitative real-time RT-PCR was performed using an RNA equivalent of 20 ng, predeveloped TaqMan assays (summarized in Table 1), TaqMan Gene Expression Master Mix, and the ViiA7 Real-Time PCR System including ViiA7 software version 1.2.2 (each from Thermo Fisher Scientific). Expression of target genes was normalized to that of 18S rRNA, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (where C_t is threshold cycle).

Immunofluorescent staining of human kidney tissue. For immunofluorescent staining of paraffin-embedded human kidney, 5-μm sections were deparaffinized by incubation in xylene, in a descending ethanol series and distilled water. Slides were cooked under pressure for 20 min in citrate buffer (pH 6.0) and incubated in ice-cold distilled water and PBS (pH 7.4). Unspecific binding was blocked by incuba-

Table 1. *Gene symbol, identifiers of TaqMan Assays (Thermo Fisher Scientific), and label dyes*

Target Gene	TaqMan Assay	Target	TaqMan Assay
NHERF-3 (PDZK1)	Hs00275727_m1,FAM	*SLC22A8* (OAT3)	Hs01056647_m1, FAM
HNF1α	Hs00167041_m1,FAM	*SLC22A11* (OAT4)	Hs00218486_m1, FAM
NR2A1 (HNF4α)	Hs00604435_m1,FAM	*SLC22A13* (OAT10)	Hs00188605_m1, FAM
SLC2A9 (GLUT9 isoform 1)	Hs01119173_m1,FAM	*SLC22A12* (URAT1)	Hs01030727_m1, FAM
SLC17A1 (NPT1)	Hs00192656_m1,FAM	*ABCC4* (MRP4)	Hs00195260_m1, FAM
SLC17A3 (NPT4)	Hs00198361_m1,FAM	18S rRNA	4319413E, VIC
SLC22A6 (OAT1)	Hs00191220_m1,FAM		

NHERF, Na^+/H^+ exchange regulatory factor; PDZK1, PDZ domain-containing 1; HNF, hepatocyte nuclear factor; NR2A1, nuclear receptor subfamily 2 group A member 1; SLC, solute carrier; GLUT, glucose transporter; FAM, 6-carboxyfluorescein; OAT, organic anion transporter; URAT1, urate transporter 1; MRP4, multidrug resistance-associated protein 4; VIC, 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein used for quantitative real-time RT-PCR.

tion with 5% FCS and 1% BSA in PBS for 1 h. For protein detection, slides were incubated at 4°C overnight with anti-PDZK1 (ab137873; Abcam, Cambridge, UK), anti-URAT1 (ab121826; Abcam), or anti-MRP4 (ALX-801-038; Enzo Life Sciences, Lausen, Switzerland) using antibody dilutions of 1:100, 1:100, and 1:20, respectively. Slides were incubated in the dark for 2 h with the respective fluorophore-coupled secondary antibody (Alexa Fluor568-conjugated goat anti-rat IgG and Alexa Fluor488-labeled chicken anti-rabbit IgG; Thermo Fisher Scientific). For mounting of samples, Roti-Mount FluorCare (Carl Roth, Arlesheim, Switzerland) containing 4′,6-diamidin-2-phenylindol was used. Immunofluorescence was visualized using the EVOS FL digital microscope (AMG).

Western blot analysis. Immunoblot detection was performed using pulverized tissues or cells. Preparation of tissue and cell lysates was performed as described previously (26). Protein samples were separated by SDS-PAGE and subsequently electrotransferred to nitrocellulose membranes using a Mini-PROTEAN Tetra Cell and a Mini Trans-Blot system (Bio-Rad, Cressier, Switzerland). The membranes were blocked with 5% FCS diluted in Tris-buffered saline containing 0.04% Tween 20 (TBS-T) at room temperature for 1 h. For protein detection, samples were incubated with the primary antibodies targeting PDZK1 (ab137873; Abcam), MRP4 (ALX-801-038; Enzo Life Sciences), URAT1 (ab121826; Abcam), HNF1α (ab96777; Abcam), β-actin (I-19: sc-1616; Santa Cruz Biotechnology, Heidelberg, Germany), or GAPDH (L-20: sc-31915; Santa Cruz Biotechnology) at dilutions of 1:2,000, 1:2,500, 1:500, 1:2,000, or 1:1,000 in TBS-T, respectively, at 4°C overnight. After incubation with the respective secondary horseradish peroxidase-coupled antibodies (each diluted 1:2,000 in TBS-T; Bio-Rad) for 1 h at room temperature, binding of the secondary antibody was visualized using chemiluminescent substrate (Pierce ECL2 Kit; Thermo Fisher Scientific), the ChemiDoc MP imaging system, and Image Lab 4.1 software (Bio-Rad).

In silico analysis of the PDZK1 promoter. The in silico search for potential HNF1α binding sites was performed using the open-source software NUBIScan (24) applying a threshold of 0.7. The matrix for the computational search was based on an evaluation of binding sites previously reported by Tronche and Yaniv and summarized in Supplemental Tables S1 and S2 (Supplemental data for this article may be found on the *American Journal of Phyiology: Renal Physiology* website.) (35).

Cloning of wild-type and mutated PDZK1 promoter fragments. A fragment comprising the base pairs −4389 to +75 of the 5′-untranslated region of the PDZK1 gene was amplified by PCR from human genomic DNA. The amplicon was subcloned into the multiple cloning site of pGL3-Basic (Promega, Dübendorf, Switzerland) using *Kpn*I and *Xho*I (Thermo Fisher Scientific). After amplification of the plasmid, shorter fragments of the promoter were generated following the cloning strategies summarized in Table 2. The sequences of the resulting plasmids were verified by Sanger sequencing (Microsynth, Balgach, Switzerland). For deletion of HNF1α binding sites the QuikChange II Multi Site-Directed Mutagenesis Kit and primers summarized in Table 2 were used.

Reporter gene assays. For cell-based reporter gene assays, 1×10^5 cells were seeded per well of a 24-well plate in 1 ml of DMEM. DMEM was replaced by 1 ml Opti-MEM (Thermo Fisher Scientific) 24 h before transfection. For transfection, Opti-MEM containing Lipofectin (6 μl), 975 ng of the indicated pGL3-basic vector variant, 25 ng of the control vector pRL-TK (Promega), and 500 ng of HNF1α-pEF6/V5-His were applied according to the manufacturer's instructions. Cells were lysed 48 h after transfection. For quantification of luciferase activities, the Dual-Luciferase Reporter Assay System (Promega) and a microplate reader (infinite 200 Pro) including the iControl software (Tecan, Männedorf, Switzerland) were used according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of *Renilla* luciferase.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (no. 9005; Cell Signaling, Allschwil, Switzerland) according to the manufacturer's instructions. For each immunoprecipitation, 25 mg of frozen and pulverized renal tissue were suspended in PBS and cross-linked with 1.5% formaldehyde. After cross-linking, 8 μg of chromatin were digested with nuclease and incubated with

Table 2. *Primers and enzymes used for cloning of PDZK1 promoter fragments into pGL3-Basic (Promega) and mutagenesis primers for deletion of HNF1α-binding sites in promoter fragment −789 to +75 bp*

Fragment, bp	Cloning Strategy
−4389 to +75	PCR using primers 5′-TTGGTACCCTCCAAGAGTGCCAAGACCAGAC and 5′-TTCTCGAGGAAGAGGAGCTGCTCTGTTCGTTC, *Kpn*I and *Xho*I
−3982 + 75	PCR using primers 5′-CCATCTTCCAGCGGATAG and 5′-GGTACCGTCGCCTCTTAAGGT, *Kpn*I and *Xho*I
−3321 + 75	PCR using primers 5′-GGTACCCACCGTGGAAAACAG and 5′-CCATCTTCCAGCGGATAG, *Kpn*I and *Xho*I
−2407 to +75	Obtained by restriction of vector containing fragment −4389 to +75 bp with *Hin*dIII
−1238 to +75	Obtained by restriction of vector containing fragment −4389 to +75 bp with *Sac*I and *Hin*dIII
−789 to +75	Obtained by restriction of vector containing fragment −4389 to +75 bp with *Nhe*I and *Hin*dIII
−789 to +75 Deletion 1	Site directed mutagenesis of vector containing fragment −789 to +75 bp using primer 5′-CAGACCACATGTCCCTGTTAAA TATTTTAAATCAATCTTTGTTCAAAG
−789 to +75 Deletion 2	Site directed mutagenesis of vector containing fragment −789 to +75 bp using primer 5′-CTTGAATTCAGACCACATGTCC CTATTTTAAATCAATCTTTGTTCAAAG

5 μg of rabbit polyclonal anti-HNF1α antibody (ab96777; Abcam) for detection of transcription factor binding, with 5 μg of normal rabbit IgG (2729; Cell Signaling) for detection of unspecific binding, and with anti-histone H3 (D2B12) XP rabbit monoclonal antibody (4620; Cell Signaling) as positive control. For detection of PDZK1 promoter sequence after immunoprecipitation, a PCR was performed using the SYBR Green PCR Master Mix (Thermo Fisher Scientific), the sense primer 5′-GCCAGAGCTTTTGGTTTGCT, and the antisense primer 5′-CTGTCTCTGCAGGTGAGCAT, generating an amplicon of 185 bp encompassing the in silico identified HNF1α motif in the PDZK1 promoter. For detection of HNF1α binding to the URAT1 promoter, the primers 5′ -AACTTAGGCCTCCCCAAGAC and 5′-TGACCGGTGACACTTATGGA were used (resulting amplicon 159 bp). The size of the PCR products was verified on a 2% agarose gel with a 100-bp GeneRuler DNA ladder (Thermo Fisher Scientific). The detection of a DNA sequence of the ribosomal protein L30 (RPL30) gene after immunoprecipitation with anti-histone H3 antibody served as positive control. The corresponding primer set was provided in the assay kit by the manufacturer (7014; Cell Signaling).

Adenoviral overexpression of HNF1α. For generation of the vector pAd-CMV-HNF1α, the coding sequence of human HNF1α (NCBI reference NM_000545.6) was transferred to the Gateway pENTR1A Dual-Selection Vector and then subcloned into pAd/CMV/V5-DEST using the Gateway BP Clonase Enzyme Mix. For production of the adenovirus (Ad), HEK293A cells were infected according to the manufacturer's instructions (Thermo Fisher Scientific). The adenoviral titer of Ad-HNF1α was determined using the Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA). To study the impact of heterologously expressed HNF1α on gene expression, RPTEC (1.6×10^4 cells/well) were infected with Ad-HNF1α for 24 h. Medium was changed, and expression of selected targets was quantified 48 h after infection.

Small-interfering RNA transfection. Knockdown of HNF1α with Silencer Select small-interfering RNA (siRNA, s13868, Ambion; Thermo Fisher Scientific) was performed by reverse transfection using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) and HNF1α-siRNA (final 30 pmol) diluted in Opti-MEM (Thermo Fisher Scientific) following the manufacturer's protocol. For transfection, 1.5×10^5 cells/well were seeded in a six-well format. For positive control, cells were transfected with Silencer Select GAPDH siRNA (Thermo Fisher Scientific). The medium was changed the next day, and 48 h after transfection total RNA was isolated.

Statistical analysis. Statistical calculations were conducted using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA). Data are presented as means ± SD. Coexpression studies were conducted performing Pearson correlation analyses. For comparison of expression in different samples, paired *t*-tests following Shapiro-Wilk normality tests were performed.

RESULTS

Coexpression of urate transporters, PDZK1, and transcription factors in human kidney. At first we verified the in situ colocalization of the scaffold protein PDZK1 with the urate transporters URAT1 and MRP4, both selected as representatives of apical urate transporters known to interact with PDZK1 (Fig. 2*A*) (2, 23). Immunofluorescent staining of human kidney sections showed not only an apical localization for both transporters in proximal tubule cells but also a significant overlap with PDZK1 (Fig. 2*A*). Western blot analysis of 21 human kidney samples revealed that protein expression of PDZK1 positively correlated with that of URAT1 and that there was a trend for correlation of PDZK1 and MRP4 expression (Fig. 2, *B* and *C*). Similar results were obtained by quantitative real-time RT-PCR (qRT-PCR) analysis, except for correlation of MRP4 and PDZK1 mRNA expression (Table 3). Analyzing the mRNA expression of other transporters summarized in the urate transportosome also revealed a significant correlation with the expression of PDZK1 in human kidney [Pearson correlation coefficient (R) ranging from 0.673 to 0.903; Table 3], thereby further supporting the notion that there is a coordinated expression of genes involved in urate transport. As mentioned before, HNF4α and HNF1α are candidates functioning as central regulators of the urate transportosome gene network. To test whether HNF1α or HNF4α may be involved in the regulation of PDZK1 expression, we analyzed and correlated the mRNA expression of these transcription factors with the expression of PDZK1 in human kidney samples. This analysis revealed a positive correlation of the mRNA of PDZK1 with that of HNF1α or HNF4α (Table 3). Because the association of HNF1α with PDZK1 expression was more pronounced, we focused on HNF1α as a modulator of the above-postulated renal gene network and further validated the correlation performing Western blot analysis. The detection of protein levels revealed that the amount of PDZK1 and HNF1α also significantly correlated ($R = 0.705$; 95% confidence interval 0.393–0.871, $P = 0.0004$; Fig. 2, *D* and *E*). To validate the hypothesis of HNF1α as a transcriptional regulator of the urate transportosome, we correlated the detected HNF1α mRNA expression with that of GLUT9, OAT1, OAT3, NPT1, NPT4, OAT4, OAT10, URAT1, and MRP4. Pearson correlation coefficients ranged between 0.740 and 0.931 (Table 3), thereby confirming the findings of previous studies and providing further evidence for our hypothesis of a balanced gene network with HNF1α as the central regulator. However, no such correlation with HNF1α was observed for the mRNA of the efflux transporter MRP4 and NPT1. When we correlated the protein expression of HNF1α with URAT1 or MRP4 as representatives of apical transporters of the renal tubule, we observed a significant correlation of URAT1 and HNF1α ($R = 0.578$; 95% confidence interval 0.195–0.808, $P = 0.0061$), and a correlation was seen for the efflux transporter MRP4 and this transcription factor ($R = 0.636$; 95% confidence interval 0.282–0.838, $P = 0.0019$).

Impact of HNF1α on PDZK1 promoter activity and mRNA expression in vitro. To validate the influence of HNF1α on transcription of PDZK1, a 4,464-bp fragment located in the 5′-untranslated region of the gene encoding for this scaffold protein was analyzed in silico for potential HNF1α-binding sites. Based on previous findings (35) and our own analysis, the most likely HNF1α response element is an everted repeat with 1 bp spacer (ER1) (Supplemental Tables S1 and S2). The in silico analysis of the promoter region revealed a total of 165 hits identifying 85 potential binding sites, of which 6 were ER1 motifs (Fig. 3*A*). Various fragments of the promoter were then tested for transactivation in cell-based reporter gene assays, showing that the transient overexpression of HNF1α augmented PDZK1 promoter activity by 4- to 13-fold, depending on the promoter fragment used for transfection (Fig. 3*A*). To assess whether the activation of the PDZK1 promoter by HNF1α was also accompanied by an altered PDZK1 mRNA expression, we performed qRT-PCR analysis, revealing that transient overexpression of HNF1α significantly induced PDZK1 mRNA expression in HeLa cells (mean ± SD of relative mRNA expression: pEF6-control 1.0 ± 0.4 vs. HNF1α 5.7 ± 3.3, $n = 4$, *t*-test, $P < 0.05$, data not shown). To char-

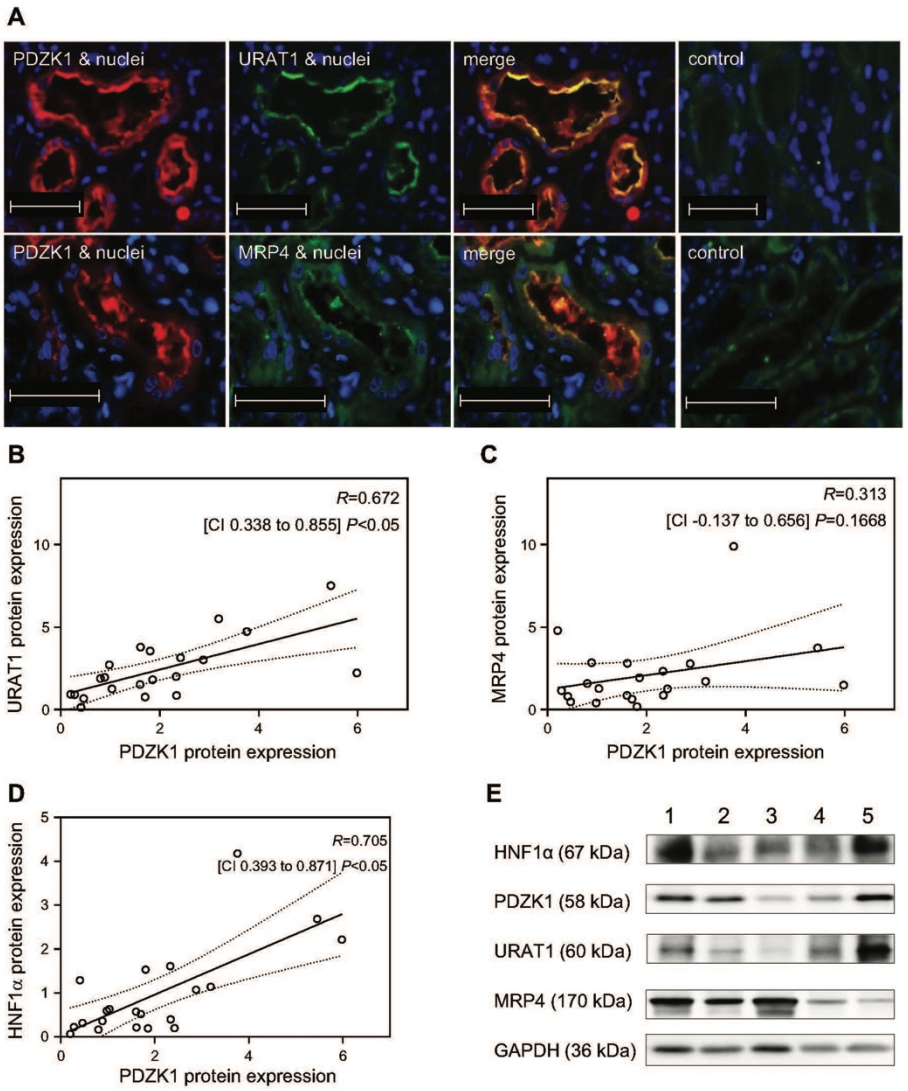

Fig. 2. Expression of urate transporter 1 (URAT1), MRP4, HNF1α, and PDZK1 in human kidney. *A*: localization of PDZK1 (red), MRP4 (green), and URAT1 (green) in human kidney was detected by immunofluorescent staining of paraffin-embedded tissue sections, revealing colocalization at the apical membrane of proximal tubule cells (yellow staining in the merged picture). 4′,6-Diamidin-2-phenylindol was used for nuclei staining (blue); scale bar: 50 μm. *B–E*: correlation of protein expression of URAT1, MRP4, and the transcription factor HNF1α with PDZK1 protein expression in human kidney samples ($n = 21$) assessed by Western blot analysis. Data were densitometrically evaluated using the Image Lab software. *R*, Pearson coefficient; CI, confidence interval. Expression normalized to that of GAPDH in the same sample was the basis of statistical analysis. *E*: representative immunoblot of proteins isolated from 5 human kidney samples. Detection of GAPDH (36 kDa) served as a loading control.

acterize the binding of HNF1α to the PDZK1 promoter, we focused on the PDZK1 promoter fragment ranging from −789 to +75 bp (Fig. 3). In silico analysis revealed the sequence 5′-GTTAAATaCATTAGC-3′ located in position −25 to −39 bp as a potential ER1 site (Fig. 3, *A–C*). The relevance of these nucleotides for transactivation of PDZK1 by HNF1α was confirmed using promoter fragments containing deletions of the ER1 site (Fig. 3*D*), showing that the wild-type promoter construct was transactivated 13-fold by HNF1α, whereas the activity of the mutated fragments was abolished,

Table 3. *Correlation of mRNA transcript number of PDZK1 or transcription factor HNF1α with expression of selected transporters of the urate transportosome in human kidney samples*

Target Gene (protein)	Correlation with PDZK1	Correlation with HNF1α
PDZK1 (NHERF-3)		$R = 0.925$ (CI 0.841–0.966); $P < 0.0001$
SLC22A12 (URAT1)	$R = 0.903$ (CI 0.795–0.955); $P < 0.0001$	$R = 0.932$ (CI 0.857–0.968); $P < 0.0001$
SLC22A11 (OAT4)	$R = 0.860$ (CI 0.714–0.935); $P < 0.0001$	$R = 0.889$ (CI 0.771–0.948); $P < 0.0001$
SLC22A13 (OAT10)	$R = 0.760$ (CI 0.534–0.885); $P < 0.0001$	$R = 0.825$ (CI 0.654–0.916); $P < 0.0001$
ABCC4 (MRP4)	$R = -0.101$ (CI −0.470 to 0.298); $P = 0.6232$	$R = -0.077$ (CI −0.444 to 0.312); $P = 0.7016$
SLC17A1 (NPT1)	$R = 0.683$ (CI 0.402–0.846); $P = 0.0001$	$R = -0.1022$ (CI −0.4642 to 0.289); $P = 0.6121$
SLC17A3 (NPT4)	$R = 0.770$ (CI 0.551–0.890); $P < 0.0001$	$R = 0.740$ (CI 0.506–0.872); $P < 0.0001$
SLC22A6 (OAT1)	$R = 0.768$ (CI 0.547–0.888); $P < 0.0001$	$R = 0.803$ (CI 0.613–0.905); $P < 0.0001$
SLC22A8 (OAT3)	$R = 0.863$ (CI 0.718–0.936); $P < 0.0001$	$R = 0.930$ (CI 0.853–0.968); $P < 0.0001$
SLC2A9 (GLUT9)	$R = 0.673$ (CI 0.394–0.839); $P = 0.0001$	$R = 0.756$ (CI 0.533–0.881); $P < 0.0001$
NR2A1 (HNF4α)	$R = 0.571$ (CI 0.243–0.781); $P = 0.0019$	$R = 0.628$ (CI 0.333–0.811); $P = 0.0003$

For assessment of mRNA expression of indicated genes quantitative real-time RT-PCR was conducted; $n = 27$ experiments. ABCC4, ATP-binding cassette transporter MRP4; NPT, Na^+-dependent phosphate cotransporter; *R*, Pearson coefficient; CI, confidence interval. For correlation analysis of transcript number of different mRNA species, transcript number was calculated using reference samples containing a known quantity of the corresponding transcripts.

thereby strongly indicating that this motif is crucial for transactivation of the PDZK1 gene by HNF1α.

Binding of HNF1α to the PDZK1 promoter in human kidney. After testing transactivation of PDZK1 promoter activity and expression in a nonrenal model, we next performed ChIP assay to test whether HNF1α binds to the PDZK1 promoter in renal tissue. Chromatin isolated from human kidney samples was cross-linked, digested, and immunoprecipitated with an anti-HNF1α antibody, showing an interaction of the transcription factor with the sequence encompassing the identified HNF1α motif in the PDZK1 promoter (Fig. 4*A*). Similar results were obtained using the same samples for detection of HNF1α binding to a fragment of the URAT1 promoter, which has previously been reported to be a target of this transcription factor (Fig. 4*B*) (13). The chromatin samples were also immunoprecipitated with an anti-histone H3 antibody for assessment of integrity of the samples. The histone H3 protein is known to be part of the nucleosome encompassing exon 3 of the RPL30 gene, which is shown and confirmed in Fig. 4*C*.

Impact of HNF1α overexpression on expression of PDZK1 in kidney cells. To validate the role of HNF1α in regulating renal PDZK1 expression, we assessed the influence of HNF1α in primary RPTEC by adenoviral overexpression of HNF1α. First, endogenous expression of prototypical renal epithelial proteins in RPTEC was verified by Western blot analysis. (Fig. 5). As shown in Fig. 6, the mRNA expression of PDZK1 was significantly enhanced when RPTEC were infected with the adenovirus encoding for HNF1α, which was confirmed by Western blot analyses (Fig. 6, *A–C*). Additionally, HNF1α significantly enhanced the mRNA expression of OAT4 (*SLC22A11*), URAT1 (*SLC22A12*), and NPT4 (*SLC17A3*), which are known interacting partners of PDZK1 (Fig. 6, *D–F*). However, no significant alteration was observed for MRP4 (*ABCC4*) (1.2 ± 1.1-fold compared to LacZ control, $P = 0.174$ by *t*-test, data not shown).

Impact of HNF1α siRNA knockdown on expression of PDZK1 in renal cells. For verification of the impact of HNF1α on PDZK1 mRNA expression, we next used siRNA to downregulate HNF1α in RPTEC and performed qRT-PCR (Fig. 6*G*). Importantly, siRNA-induced downregulation of HNF1α was accompanied by a significant decrease in expression of PDZK1 (Fig. 6*H*), whereas mRNA expression of URAT1 (mock vs. HNF1α siRNA: 100.0 ± 3.6 vs. 120.6 ± 33.2, $n = 4$, $P = 0.356$ by *t*-test, data not shown) and MRP4 (100.0 ± 3.1 vs. 106.7 ± 13.9, $P = 0.5379$ by *t*-test, data not shown) was not significantly altered.

DISCUSSION

Previous studies show that most of the apical transporters of the proximal tubule directly interact with the membrane-associated adaptor protein PDZK1, which is localized in the brush border of epithelial cells and is capable of regulating function and subcellular localization of the interacting proteins (9, 12). However, little is known about the mechanisms synchronizing the interplay of renal transporters and the posttranslational modulator PDZK1. Based on the observed coexpression of PDZK1 and of selected apical membrane transporters, we hypothesized that there should be a mechanism coordinating the expression of proteins involved in renal uptake and efflux. One mechanism known to integrate the function of gene networks is the action of transcription factors (34). Supported by findings in HNF1α-deficient mice in which dysfunctional transport in the renal proximal tubule cells results in polyuria, glucosuria, and aminoaciduria, the homeobox protein may be considered as a key determinant of proximal tubular function (25). Indeed, this homeobox protein has been described for modulation of various members of the renal transportosome (11, 13, 14, 16, 31). Hence, to provide evidence that PDZK1 is part of a coordinated transcriptional network summarizing the urate transportosome, we tested whether HNF1α also modulates transcription of PDZK1.

Our results show that PDZK1 mRNA and protein expression significantly correlated with that of HNF1α in human kidney, supported by our findings revealing that the homeobox protein enhances PDZK1 promoter activity in cell-based reporter gene assays and upregulates PDZK1 mRNA and protein expression in renal proximal tubule epithelial cells. The transcriptional regulation of PDZK1 by HNF1α was further verified by siRNA-mediated knockdown of HNF1α. Reduction of HNF1α significantly diminished PDZK1 mRNA expression levels in RPTEC. The moderate downregulation of PDZK1 suggests that alternative pathways of transcriptional regulation are present in those cells. In this regard, regulation of PDZK1 by the nuclear receptors PPARα and HNF4α has previously been shown in hepatocytes (3, 4, 33, 37).

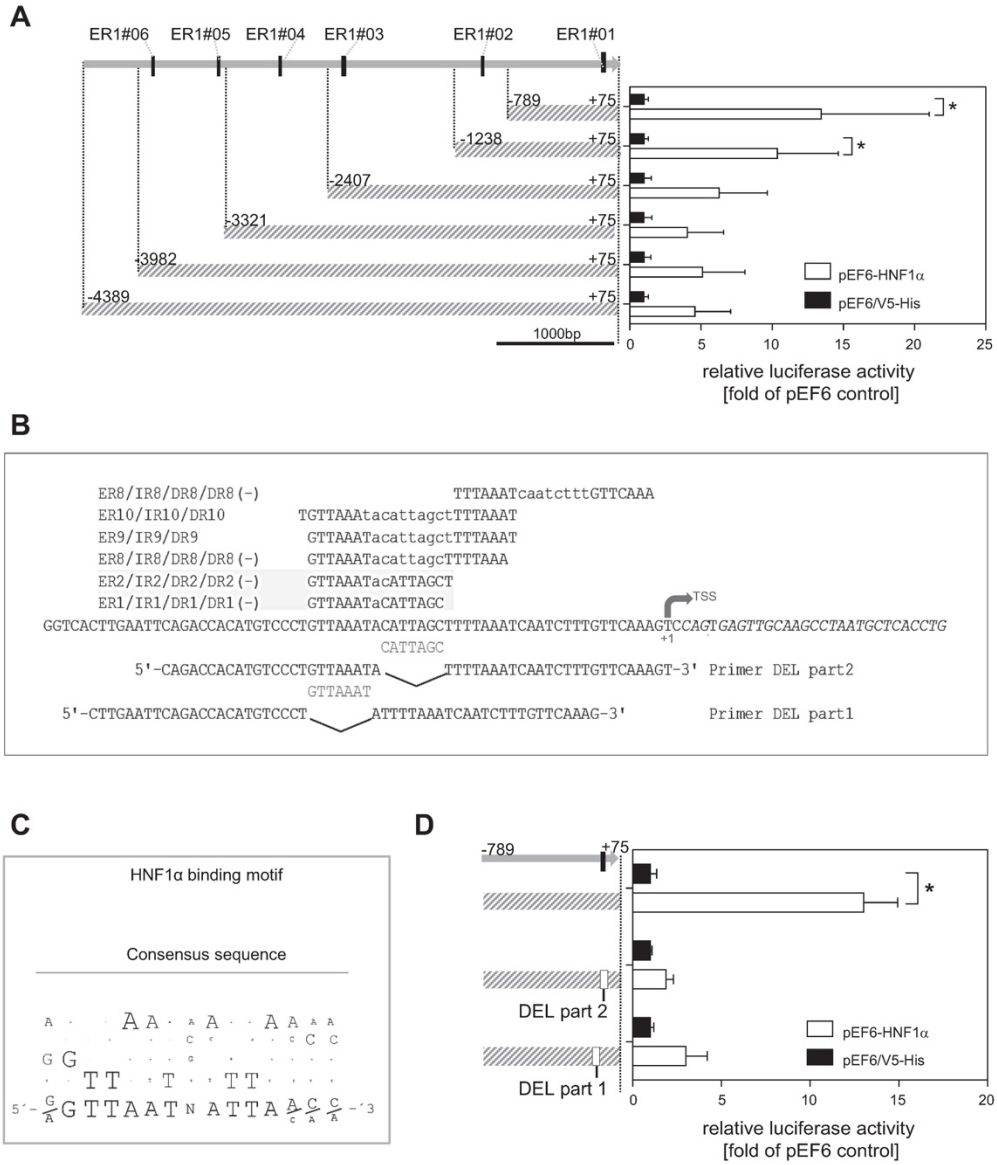

Fig. 3. Characterization of the PDZK1 promoter harboring several HNF1α-binding motifs. ER, everted repeat. *A*: promoter activity assessed by dual luciferase reporter gene assay using indicated PDZK1 promoter reporter gene constructs in the absence or presence of expression vector harboring the coding sequence of HNF1α (pEF6-HNF1α). Empty vector pEF6/V5-His served as the DNA loading control. Data are means ± SD (ratio of firefly and *Renilla* luciferase activity normalized to cells cotransfected with PDZK1 promoter fragment and pEF6/V5-His; $n = 3$, 2-way ANOVA pEF6/V5-His vs. pEF6-HNF1α for each promoter construct, Bonferroni posttest, $^*P < 0.005$). *B*: in silico analysis of HNF1α-binding site ER1#01 near the transcription start site TSS of the PDZK1 gene (NCBI NM_001201325.1). *C*: the consensus sequence of HNF1α-binding motif based on the literature was used as a matrix for NUBIScan analysis. *D*: impact of deletion of an in silico identified HNF1α-binding motif on PDZK1 promoter activity in HeLa cells. Activity of mutated PDZK1 promoter constructs (DEL, deletion of nucleotides in ER1#01) in the presence and absence of pEF6-HNF1α was assessed by dual luciferase reporter gene assay. Data are means ± SD (ratio of firefly and *Renilla* luciferase activity normalized to cells cotransfected with PDZK1 promoter fragment and pEF6/V5-His; $n = 3$, 2-way ANOVA pEF6/V5-His vs. pEF6-HNF1α for each promoter construct, Bonferroni posttest, $^*P < 0.005$).

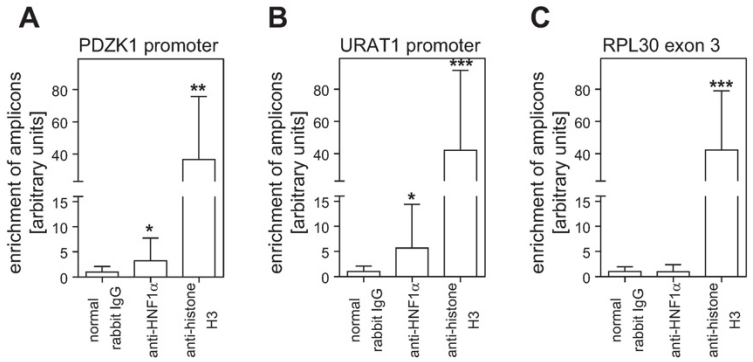

Fig. 4. Results of chromatin immunoprecipitation of protein-DNA complexes in human kidney tissue. Enrichment of DNA after immunoprecipitation with indicated antibodies was quantified by real-time PCR. Data are means ± SD ($n = 4$, ratio-paired t-test vs. rabbit IgG, $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$). The resulting threshold cycle (C_t) values were normalized to the C_t value of the corresponding chromatin input control, and relative expression was calculated according to the $2^{-\Delta\Delta C_t}$ method. *A* and *B*: detection of HNF1α binding to the PDZK1 and URAT1 promoter by chromatin immunoprecipitation with rabbit anti-HNF1α antibody. Normal rabbit IgG was used for detection of unspecific binding. Rabbit anti-histone H3 antibody was used for detection of histone H3-DNA complexes. *C*: detection of histone H3 protein bound to the ribosomal protein L30 (RPL30) gene served as control for integrity of the samples.

Moreover, it has to be considered that, even if the herein used RPTEC exhibit a preserved renal epithelial phenotype expressing multiple transporters, HNF1α mRNA expression in these cells was quite low but reliably detectable. In contrast, HNF1α protein expression in native cells was below the limit of detection in our experiments. However, we consider the response of PDZK1 transcription to the siRNA-mediated knockdown as an additional indicator for the link between HNF1α and PDZK1 transcription. Importantly, because of the low expression of HNF1α in the herein used cellular model, the lack of transcriptional response as observed for URAT1 and MRP4 in the same experiment should not be considered as proof for a missing link between HNF1α and their transcription.

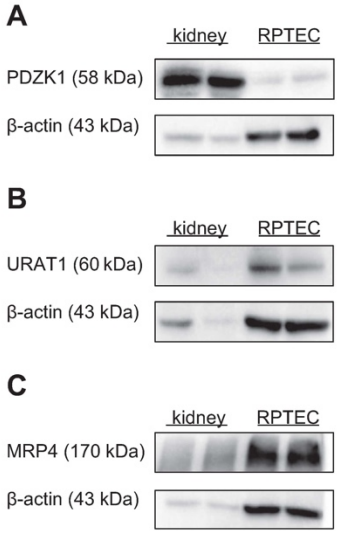

Fig. 5. Western blot analysis for assessment of protein expression of PDZK1, MRP4, and URAT1 in renal proximal tubule epithelial cells (RPTEC). Expression was assessed in native RPTEC (2 passages) and commercially obtained whole cell lysates from human kidney (AMS biotechnology). Detection of β-actin served as a loading control.

However, the idea that HNF1α is not only a regulator of PDZK1 but also of the transporter network in the human proximal tubule was strengthened by our correlation studies in human kidney samples, revealing an association of mRNA expression of HNF1α with that of the apical renal transporter URAT1, which has been previously reported to be an interacting partner of PDZK1 and a target gene of HNF1α (5, 13). Accordingly, our results showed that the mRNA expression level of URAT1 increased significantly following HNF1α overexpression in RPTEC. Notably, in human kidney samples, HNF1α mRNA expression similarly correlated with the expression of apically expressed NPT4, OAT4, and OAT10 and with that of the basolaterally expressed transporters OAT1, OAT3, and GLUT9, of which at least NPT4, OAT4, OAT1, and OAT3 have been reported as HNF1α target genes (5, 11, 16, 29, 31). These findings indicate that the impact of HNF1α is not limited to proteins interacting with PDZK1 and to transporters at the luminal pole of the tubule cell. Hence, it may be assumed that HNF1α balances the expression of the members of the transportosome on the whole and modulates PDZK1 expression while PDZK1 stabilizes the apical localization of selected transporters.

Even if our analysis of protein expression in human kidney revealed a correlation of MRP4 with HNF1α, our findings from the correlation analyses in human kidney samples revealed no association of HNF1α mRNA expression with that of MRP4. Moreover, we observed no influence of HNF1α on MRP4 mRNA expression after adenoviral-mediated upregulation of this transcription factor in cultured RPTEC. Focusing on regulation of PDZK1 expression, we did not further investigate this observation and are not able to explain the discrepancy between protein and RNA data. Contradictory results have also been described by Maher et al. who analyzed the impact of HNF1α in a knockout model, showing that loss of this transcription factor is associated with upregulation of

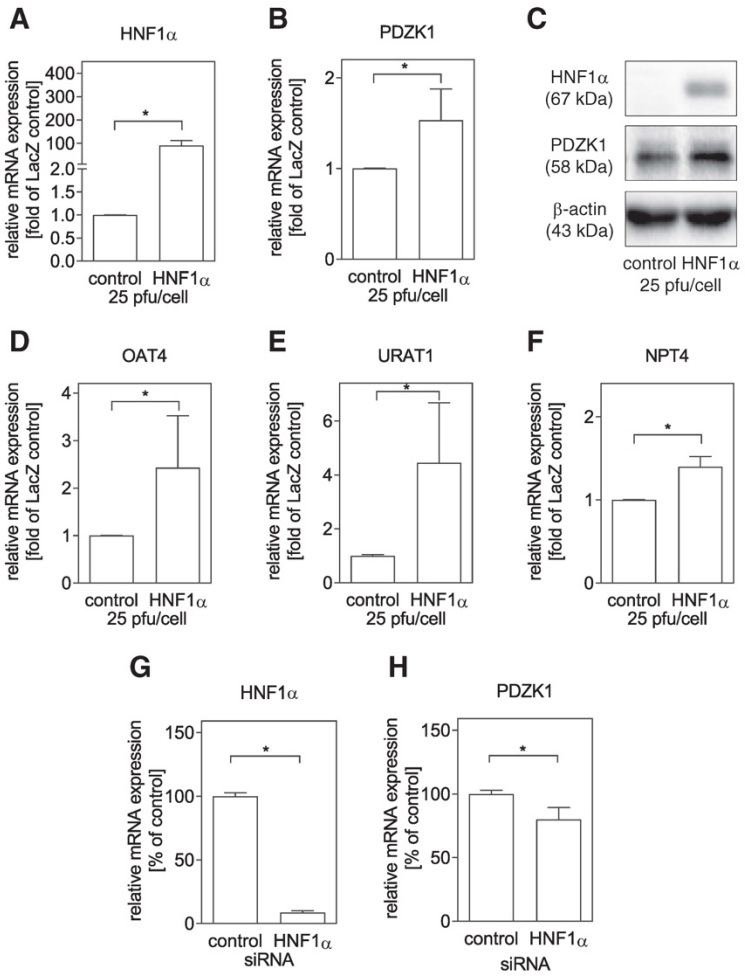

Fig. 6. Impact of adenoviral overexpression of HNF1α on expression of PDZK1 and selected members of the urate transportosome in a renal cell model. *A*, *B*, and *D–F*: the impact of exogenously induced HNF1α on endogenous mRNA expression of selected targets was assessed by quantitative real-time RT-PCR 48 h after infection of RPTEC with 25 plaque-forming units (pfu)/cell (LacZ control vs. HNF1α). Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. Data are means ± SD ($n = 4$, *t*-test, $^*P < 0.05$). *C*: representative Western blot analysis for assessment of protein expression of PDZK1 and HNF1α in whole cell lysates of RPTEC after infection with 25 pfu/cell of HNF1α virus or LacZ control. Detection of β-actin served as a loading control. *G* and *H*: the influence of reduction of HNF1α by small-interfering RNA (siRNA, 48 h) on endogenous mRNA expression was assessed by quantitative real-time RT-PCR. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. Data are means ± SD ($n = 4$, *t*-test, $^*P < 0.05$).

Mrp4 in the murine kidney and liver, but not in the duodenum (16), indicating that this transporter may be regulated by an additional factor.

However, our data suggest that HNF1α may be a key determinant of the coordinated expression of the genes summarized in the urate transportosome. Because the human kidney is an organ with high eliminative capacity, one might speculate that interindividual differences in HNF1α and PDZK1 expression in renal tissue might affect membrane transport, and thereby interindividual differences in physiological parameters such as uric acid levels and pharmacokinetics. Remarkably, the impact of PDZK1 on hepatic pharmacokinetics has already been demonstrated in *Pdzk1−/−* knockout mice. In detail, this study demonstrated that loss of Pdzk1 is accompanied with a reduced hepatic elimination of sulfobromophthalein, which is a prototypical substrate of organic anion transporting protein 1a1, which in turn is an interacting partner of Pdzk1 (38). Thus, taking the regulatory role of HNF1α in PDZK1 expression and membrane transport into account, it is of interest whether drugs in clinical use or exposure to environmental toxins influences expression of this transcription factor, thereby modulating cellular handling of drugs.

Taken together, we hypothesized that PDZK1, as a post-translational modulator of transporters expressed at the apical tubular membrane, is part of a coordinated gene network that summarizes renal membrane transporters, thereby synchronizing the regulation of renal tubule function. Our results demonstrate that homeobox protein HNF1α, which is also assumed to contribute to renal developmental expression of drug transporters, controls the expression of PDZK1.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.P., J.H., and H.E.M.z.S. conceived and designed research; K.P., J.H., C.F., I.S., V.B., U.Z., and H.E.M.z.S. performed experiments; K.P., J.H., C.F., I.S., V.B., U.Z., and H.E.M.z.S. analyzed data; K.P., J.H., C.F., I.S., V.B., U.Z., and H.E.M.z.S. interpreted results of experiments; K.P., J.H., and H.E.M.z.S. prepared figures; K.P., J.H., and H.E.M.z.S. drafted manuscript; K.P., J.H., C.F., I.S., V.B., U.Z., and H.E.M.z.S. edited and revised manuscript; K.P., J.H., and H.E.M.z.S. approved final version of manuscript.

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3.2 The nuclear receptors PXR and LXR are regulators of the scaffold protein PDZK1

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Abstract

PDZK1 (NHERF3) interacts with membrane proteins whereby modulating their spatial arrangement, membrane stability, and function. One of the membrane proteins shown to be stabilized by interaction with PDZK1 is the HDL-receptor SR-BI (SCARB1), whereby suggesting a role of PDZK1 in cholesterol homeostasis. Accordingly, the influence of TO 901317, a known activator of liver X receptor alpha (LXRα, NR1H3) which is a central regulator of the lipid homeostasis, on PDZK1 and SR-BI was tested in mice. Administration of TO 901317 did not affect PDZK1 expression and reduced the amount of SR-BI protein in mouse liver. Considering that TO 901317 also activates the xenosensor pregnane X receptor (PXR, NR1I2), it was aim of this study to further investigate their influence on PDZK1.

First, we tested the transactivation of PDZK1 by LXRα or PXR in cell-based reporter gene assays comparing the effect of prototypical ligands to that of TO 901317. Ligand mediated activation of LXRα increased, while that of PXR lowered luciferase activity. Further, we located the most likely binding site for LXRα on the PDZK1 promoter between the -69bp to -54bp, while PXR seemed to indirectly interact with the promoter. The transcriptional regulation by LXRα was further supported showing enhanced mRNA expression of PDZK1 in HepG2 cells treated with the selective LXRα-agonist GW3965, while treatment with TO 901317 reduced the protein amount of PDZK1.

Taken together, we provide evidence that both LXRα and PXR are transcriptional regulators of PDZK1, whereby suggesting that the scaffold protein is part of cholesterol homeostasis and drug metabolism.

Keywords

Scaffold protein, PDZ domain, PDZK1, LXR, PXR, HDL

Introduction

The protein family of Na^+/H^+ exchanger regulatory factors (NHERF) summarizes four members all shown to modulate localization and function of membrane proteins [1]. This function is linked to PDZ-domains, a common feature of the NHERF protein family. In detail, PDZ-domains recognize and bind to specific amino acid sequences at the C-terminus of their target proteins, whereby modulating the localization, spatial arrangement, and/ or stability of the interacting protein [2]. One NHERF is the PDZ domain containing protein 1 (PDZK1), also known as NHERF3. This scaffold protein has been investigated for its role in various physiological and pharmacological functions. One of these functions is the modulation of uptake and efflux transporters involved in uric acid handling at the apical membrane of the renal tubular cells. In detail, direct interaction of PDZK1 was shown to enhance membrane localization and function of members of the solute carrier superfamily (SLC; e.g. SLC22A11, OAT4) and the ATP-binding cassette superfamily (ABC; e.g. ABCC4, MRP4) [3, 4], which are summarized in the "urate transportosome". This translated into the assumption that PDZK1 orchestrates the spatial arrangement of the transporters, which are part of this particular multi-protein complex [5]. In the context of PDZK1 being involved in the functionality of the "urate transportosome" it seems noteworthy that we recently showed PDZK1 being transcriptionally regulated by HNF1α [6]. Considering that several members of the "urate transportsome" are also transcriptionally modulated by this homeobox domain factor suggests, that PDZK1 is part of a coordinated gene network with functional importance in renal uric acid clearance. Furthermore, the scaffold protein has been shown to be transcriptionally regulated by thyroid hormone receptor alpha (THRα, NR1A1) and beta (THRβ, NR1A2) [7], which coordinate the activity of a gene network involved in various metabolic pathways. In the THR-modulated gene network, PDZK1 may regulate the transmembrane transport of thyroid hormones or the activity of other membrane proteins. Indeed, the spectrum of proteins interacting with and functionally modulated by PDZK1 is not limited to transport proteins, but has also been shown for receptors such as the serotonin 2A receptor (HTR2A) and the scavenger receptor B type I (SR-BI; *SCARB1*) [8, 9]. The latter is a membrane receptor mediating cellular uptake of lipids from high density lipoproteins (HDL) in liver and steroidogenic tissues [10]. HDL particles are assumed to be cardio-protective due to their ability to transport excess cholesterol from the periphery to liver [11]. In 2002, David Silver reported that deletion of the terminal leucine of SR-BI significantly influences *in vivo* cell surface expression in hepatocytes of transgenic mice [12]. This observation is in accordance with findings by Kocher *et al.* showing that deletion of *Pdzk1* in mice results in reduced expression of SR-BI in liver and intestine, thereby explaining the previously reported changes in HDL-particle size and cholesterol plasma level in this mouse model [13, 14]. These findings can be related to the function of SR-BI as

mediator of the HDL endocytosis and the ability to influx the cholesteryl ester from HDL without metabolizing the particle itself [15]. Data by Yesilaltay *et al.* comparing *Pdzk1*$^{-/-}$ and *Sr-b1*$^{-/-}$ mice suggest that the function of the scaffolding protein can rather be seen in a membrane stabilization of the HDL-receptor preventing cellular degradation than in a change of cellular sorting [16]. Indeed, changes in Pdzk1 directly influence the function of SR-BI. This was shown in mice where decreased hepatic levels of phosphatidylcholine (in mice lacking phosphatidylethanolamine N-methyltransferase) stimulated transcription and thereby protein expression of Pdzk1. Even if the transcription of the scavenger receptor (SR-BI receptor) was unchanged, the protein expression increased resulting in a significant decrease of the HDL cholesterol plasma levels. Noteworthy is, that the augmentation of SR-BI expression correlated with that of PDZK1 [17].

Another factor known to regulate handling of cholesterol in the organism is the activation of the liver X receptors, LXR α (NR1H3) and LXR β (NR1H2) [18]. LXRs are ligand activated transcription factors functioning as intracellular sensors of excess cholesterol and regulating adaptive processes protecting the cell from cholesterol overload. Indeed, activation of LXRs results in the transcriptional activation of a network of genes involved in reverse cholesterol transport, whereby enhancing cholesterol removal from the cell, transport to the liver and finally biliary excretion [18]. Furthermore, activation of LXRs reduces intestinal cholesterol absorption, cholesterol synthesis, and uptake by the cell [19]. In accordance with the function of SR-BI as mediator of hepatic lipid uptake, this receptor has been shown to be significantly induced treating HepG2 cells with the oxysterol 22R-OH-cholesterol, a known activator of LXR [20]. Hypothesizing that due to its impact on SR-BI, PDZK1 should be part of the LXR-regulated gene network, Grefhorst *et al.* tested the impact of prolonged pharmacological treatment with the LXR-agonist TO 901317 on Pdzk1 and Sr-b1in mice. Surprisingly, even if the treatment significantly increased HDL particle size and plasma levels, the authors observed a significantly reduced amount of Sr-b1 in the membrane, while a smaller most likely "immature" SR-BI variant appeared. Furthermore, they found no change in hepatic expression of Pdzk1 in treated mice. According to their findings, changes in reverse cholesterol transport with enhanced whole body cholesterol removal are independent of SR-BI and Pdzk1 [21].

Importantly, TO 901317 is not only an activating ligand of LXRs, but also of the human nuclear receptor pregnane X receptor (PXR, NR1I2) [22]. The latter is well known as xenosensor, balancing xenobiotic exposure and activity of genes involved in detoxification and metabolism by transcriptional activation of metabolizing enzymes (e.g. CYP450) and drug transporters (e.g. ABCC2) in human liver [23, 24]. However, the role of PXR is not limited to drug

metabolism, as several studies reported an involvement in endocrine homeostasis, energy management or inflammation [24-26].

It was aim of this study to investigate the regulation of PDZK1 by LXR and PXR to clarify the role of both nuclear receptors in the regulation of PDZK1. Therefore we preformed cell-based reporter gene assays as well as mRNA and protein expression analyses comparing TO 901317 and GW3965, the latter being a selective LXR agonist [27].

Material and Methods

Cell culture & chemicals

HepG2 cells (ATCC® HB-8065™) were cultured at 37°C in a humidified 5% CO_2-atmosphere. Dulbecco's Modified Eagle's Medium (D6429, 4.5 mg/L glucose and 1 mM sodium pyruvate, Sigma-Aldrich, Arlesheim, Switzerland) supplemented with 10 % Fetal Calf Serum (FCS; AMIMED, BioConcept Ltd., Allschwil, Switzerland) and 1% GlutaMAX™ (Thermo Fisher Scientific, Reinach, Switzerland) was used as culture medium. The LXR agonist GW3965 and the PXR agonist rifampicin were purchased from Sigma Aldrich while the PXR/LXR agonist TO 901317 was obtained from Tocris (Zug, Switzerland).

Plasmids

The generation of the luciferase reporter vectors (pGL3-basic, Promega, Dübendorf, Switzerland) containing the −4386 bp to +75 bp fragment of the 5'UTR of PDZK1 or 5'-deletion variants of the promoter was described elsewhere [7]. The most likely binding site for LXRα (NR1H3) and PXR (NR1I2) was deleted by Overlap Extension PCR [28] using the primers GLprimer2 5'-CTTTATGTTTTTGGCGTCTTCCA-3', RVprimer3 5'-TAGCAAAATAGGCTGTCCC-3', Del_For 5'-GGACAGGACCACATGTCC-3', and Del_Rev 5'-GTGGTCCTGTCCTGCTTC-3' (MicroSynth AG, Balgach, Switzerland), and the -789 bp to +75 bp PDZK1promoter-pGL3-basic as template. The reporter gene vectors CYP3A4-XREM-pGL3-basic or SHP1promoter-pGL3-basic and the expression vectors PXR (NR1I2)-pEF6, LXRα (NR1H3)-pEF6 and RXRα (NR2B1)-pEF6 have previously been reported [29-32].

Cell-based reporter gene assays

Transactivation of PDZK1 by LXRα or PXR was investigated performing cell-based reporter gene assays. 80,000 HepG2 cells per well were seeded in 24-well plates and transfected using jetPRIME® (Polyplus, Chemie Brunschwig, Basel, Switzerland). The ratio of µg DNA to µl transfection reagent was 1:3. Each well was transiently transfected with 25 ng pRL-TK (Promega) encoding for *Renilla* luciferase, 250 ng of the respective pEF6 expression vector, and 500 ng of the respective promoter construct in pGL3-basic. After 4 hours, the medium was changed. The day after transfection, cells were treated with the respective compound (GW3965, TO 901317 or rifampicin) or solvent control (DMSO). After 24 hours of treatment, the cells were lysed in 120 µl lysis buffer (Promega). 20 µl lysate were transferred into white 96-well plates (Greiner, Huberlab, Aesch, Switzerland) to quantify the activity of *Renilla* and *Firefly* luciferase using the Dual-Luciferase® Reporter Assay System (Promega). Luminescence was detected with the Infinite® M200 Pro (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions. The *Firefly* luciferase activity was normalized to that of *Renilla* in each sample.

***In silico* scan for transcription factor binding motifs**

The open access program NUBIScan version 2.0 (www.nubiscan.unibas.ch) was applied to search for potential LXRα or PXR binding sites. The underlying algorithm has previously been described and published [33]. The fragment used was the -4386 bp to +75 bp of the 5'UTR of the gene encoding for PDZK1 (NM_002614.4). The search matrix was based on previously described data for LXRα and PXR response elements [34-36], and the search parameter threshold was set to 0.6.

Real Time-PCR

Real-time PCR was performed to determine the influence of 10 µM TO 901317, 10 µM GW3965, or 10 µM rifampicin on the amount of *SCARB1* or of *PDZK1* mRNA. Briefly, HepG2 were seeded in 12-well plates, after 48 hours of treatment total mRNA was extracted using pegGold RNAPure™ (Axon Lab, Baden-Dättwil, Switzerland) according to the protocol of the manufacturer. 1 µg of RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (LuBioScience, Zürich, Switerland), followed by expression analysis of *SCARB1*, *PDZK1*, and *18S* ribosomal RNA (18S) using commercially available TaqMan® gene expression assays (Hs00969821_m1, Hs00275727_m1, and Hs4319413E; Applied Biosystems, Thermo Fisher Scientific, Reinach, Switzerland). The

samples were analyzed using the ViiA™ 7 Real-Time PCR System. Expression was calculated applying the $2^{-\Delta\Delta CT}$ method as previously described [37].

Western Blot analysis

Protein expression of PDZK1 and SR-BI was analyzed by immunoblotting. Briefly, 3 x 10^5 HepG2 cells per well were seeded on 6-well plates. The day after seeding, the cells were treated with 10 µM GW3965, 10 µM TO 901317, 10 µM rifampicin, or solvent control (DMSO) for 48 hours. After that, cells were lysed in 5 mM Tris-HCl (pH 7.4) and disrupted by five freeze-thaw cycles. 10 ug of protein were separated by sodium dodecyl sulfate - 7.5 % polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane using a tank blotting system (Mini Trans-Blot® Cell; Bio-Rad Laboratories, Cressier FR, Switzerland). Prior to incubation with the respective primary antibody at 4°C overnight, the membranes were blocked with 5 % FCS (AMIMED) diluted in Tris-buffered saline (TBS) supplemented with Tween 20 (TBS-T, Carl Roth; Karlsruhe, Germany). For detection of PDZK1, SR-BI, and β-actin the rabbit monoclonal anti-PDZK1 antibody ab92491 (Lucerna-Chem, Lucerne, Switzerland; diluted 1:2500), the rabbit polyclonal anti-SR-BI antibody ab106572 (Lucerna-Chem, diluted 1:750), and goat polyclonal anti-β-actin antibody sc-1616 (Santa Cruz Biotechnology, Heidelberg, Germany; diluted 1:1000) were used, respectively. Secondary horseradish peroxidase (HRP) -conjugated antibodies (Bio-Rad Laboratories; diluted 1:2000) were applied to detect the immobilized primary antibody. The immobilized HRP-conjugated secondary antibodies were visualized with the Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific). The ChemiDoc™ MP System (Bio-Rad Laboratories) running with the Image Lab™ Software 4.1 (Bio-Rad Laboratories) was used to digitalize and analyze the data.

Statistical analysis

GraphPad Prism software, version 6 (GraphPad Software, San Diego CA, U.S.A.) and Microsoft Excel (Microsoft, Redmond WA, U.S.A.) were used for data analysis. $p^* \leq 0.05$ was considered as statistically significant. Data are presented as mean ± standard deviation (SD).

Results

Ligand activated LXRα enhances PDZK1 promoter activity

At first, we tested the transactivation of the PDZK1 promoter by LXRα in cell-based luciferase assays. In the human hepatoma cell line (HepG2) transfected with the promoter construct (PDZK1-promoter-pGL3basic) and the LXRα

expression vector (LXRα–pEF6) luciferase activity significantly increased after treatment with 10 μM TO 901317 (mean normalized luciferase activity ± SD; DMSO vs. TO 901317, 1.00 ± 0.06 vs. 2.86 ± 0.80, n = 3, p-value < 0.05 unpaired t-test, fig. 1 A). A similar induction was observed using 10 μM GW3965 as activating ligand (DMSO vs. GW3965, 1.00 ± 0.23 vs. 1.57 ± 0.22, n = 3, p-value < 0.05 unpaired t-test, fig. 1 C). The SHP1-promoter-pGL3basic served as control for the activation of LXRα [31].

The nuclear receptor LXRα binds to its response element as heterodimer with RXRα [38]. For this reason, HepG2 cells were co-transfected with LXRα and its dimerization partner showing an even higher luciferase activity after treatment with GW3965 (mean normalized luciferase activity ± SD; LXRα vs. LXRα/RXRα, 1.57 ± 0.22 vs. 2.70 ± 0.21, n = 3; p-value < 0.05 by unpaired t-test, fig. 1 F). In contrast, treatment of LXRα/RXRα co-transfected HepG2 cells with TO 901317 significantly reduced transactivation compared to transfection without the dimerization partner of LXRα (LXRα vs. LXRα/RXRα, 2.86 ± 0.80 vs. 1.31 ± 0.13, n = 3; p-value < 0.05 by unpaired t-test, fig. 1 G). This indicated that TO 901317 activates another endogenous dimerization partner of RXRα, whereby preventing transactivation of PDZK1 by LXRα.

Repression of PDZK1 promoter activity after activation of PXR

As mentioned before, Mitro *et al.* reported that TO 901317 not only activates LXRα, but also PXR which also dimerizes with RXRα prior to binding to its response elements [39]. In order to verify the activation of PXR, and to test whether there is an interaction with the PDZK1 promoter HepG2 cells were exposed to TO 901317. CYP3A4-XREM-pGL3basic served as control [40]. Exposure of PXR transfected HepG2 to TO 901317 significantly repressed luciferase activity by 85% (mean normalized luciferase activity ± SD, DMSO vs. TO 901317, 1.00 ± 0.28 vs. 0.15 ± 0.05, n = 3; p-value < 0.05 by unpaired t-test, fig. 1 B), while there was no change in presence of GW3965 (DMSO vs. GW3965, 1.00 ± 0.43 vs. 0.70 ± 0.25, n = 3; p-value < 0.05 by unpaired t-test, fig. 1 D). Treatment with rifampicin, the prototypical PXR agonist [40], similarly reduced promoter activity compared to solvent control (mean normalized luciferase activity ± SD; DMSO vs. rifampicin, 1.00 ± 0.09 vs. 0.33 ± 0.04, n = 3; p-value < 0.05 by unpaired t-test, fig. 1 E).

***In silico* analysis for identification of potential LXR and PXR binding motifs on the PDZK1 promoter**

The change of PDZK1 promoter activity by LXRα and PXR indicated that both nuclear receptors are involved in transcriptional regulation of the scaffold protein. To delimit the potential binding site, the enhancer region was analyzed for the following motifs: DR4 (direct repeat 4 bp spacer), IR1 (inverted repeat 1 bp spacer) and IR0 (inverted

repeat 0 bp spacer), which are known to be shared by LXR and PXR. The *in silico* analysis of the sequence with the NUBIscan software identified eight potential binding sites within the tested promoter fragment (table 1, fig. 2A). Next, we tested transactivation of six 5' deletion fragments of the PDZK1 promoter revealing that the LXRα/RXRα-mediated (fig. 2 B) and PXR-mediated (fig. 2 C) change in luciferase activity remained until the -789 bp to +75 bp pGL3-PDZK1-promoter fragment. However, in this fragment of the PDZK1 promoter only one DR4 motif (AGGTCActtgAATTCA) located at position -69 bp to -54 bp was identified by the *in silico* analysis.

Deletion of the calculated LXR and PXR binding site

Considering the most likely binding site location of LXRα and PXR in the -789 bp to +75 bp fragment of the PDZK1 promoter, we deleted the only DR4 motif located within this fragment. Deletion of the DR4 motif significantly decreased transactivation by LXRα compared to the wild type (mean normalized luciferase activity ± SD; wild type vs. deletion, 2.53 ± 0.22 vs. 1.76 ± 0.41, n = 3, p-value < 0.05 by two-way ANOVA with Sidak's multi-comparison test). However, no change was noticed for the influence of PXR on the PDZK1 promoter. This observation suggested that either the PXR effect is of indirect nature or that PXR interacts with an additional binding motif, which was not included in the herein performed *in silico* analysis.

Analysis of mRNA and protein expression in HepG2 after exposure to TO 901317, GW3965 and rifampicin

In the reporter gene assays TO 901317, GW3965 and rifampicin influenced the PDZK1 promoter activity. To test whether these compounds affect mRNA expression of *PDZK1* and *SCARB1*, HepG2 cells were treated for 48 hours with 10 µM of TO 901317, GW3965 or rifampicin. HepG2 cells exposed to GW3965 exhibited an increased *PDZK1* mRNA expression (normalized *PDZK1* mRNA expression; mean ± S.D.: 1.00 ± 0.01 vs. 2.36 ± 0.44, fig. 4 A, p = 0.048), while TO 901317 and rifampicin did not affect the mRNA amount. Although none of the tested compounds significantly influenced transcription of *SCARB1* whereas a trend to higher mRNA levels was observed after exposing HepG2 cells with GW3965 (*SCARB1* mRNA expression normalized to DMSO, mean ± S.D.: 1.00 ± 0.02 vs.1.92 ± 1.00, fig. 5 A, p = 0.06). Considering that mRNA expression not always correlates with the amount of protein, we investigated whether treatment influences that of PDZK1 and SR-BI, respectively. Interestingly, exposition of HepG2 cells to TO 901317 resulted in a 50% reduction of PDZK1 (PDZK1 protein expression normalized to DMSO, mean ± S.D.: 1.00 ± 0.22 vs. 0.51 ± 0.11, n = 3, fig. 4 B, p = 0.042), while SR-BI was not affected. The increased PDZK1 mRNA amount after exposing HepG2 cells to GW3695 did not correlate with a higher protein amount of the scaffold

protein and also the treatment with rifampicin seemed to reduce the protein expression of PDZK1 and SR-BI, but ultimately none of them did significantly differ from control.

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Discussion

Reasoned by the crucial role of PDZK1 in lipid homeostasis where the scaffold protein stabilizes the HDL-receptor SR-BI at the plasma membrane [41-43] it has been hypothesized that PDZK1 is transcriptionally regulated by the lipid sensor liver X receptor (LXRα, NR1H3). However, in 2012 no change in Pdzk1 expression was observed in mice treated with TO 901317, whereby suggesting that the assumption of PDZK1 being part of the LXR-mediated gene network was wrong [21]. Interestingly, the non-steroidal LXR agonist TO 901317 has previously been reported to also activate the xenosensor pregnane X receptor (PXR, NR1I2) [44]. Hence, we focused on the direct interaction of LXRα and of PXR with the human PDZK1 promoter assessing transactivation in cell-based luciferase assays. Comparing the influence of GW3965 and TO 901317 revealed significant promoter activation in HepG2 cells transfected with LXRα. However, in presence of the dimerization partner retinoid X receptor α (RXRα, NR2B1), LXRα-mediated transactivation by GW3965 was significantly enhanced, while the influence of TO 901317 on the PDZK1 promoter was abolished. Determining luciferase activity after transfection of PXR and treatment with this compound or the prototypical PXR-ligand rifampicin revealed significant repression compared to solvent control, thereby suggesting, that PXR itself affects transcription of PDZK1.

As mentioned above, LXRα and LXRβ function as regulators of cholesterol and lipid homeostasis, protecting the body from cholesterol excess enhancing the expression of genes involved in reverse cholesterol transport (RCT) and in the trans-intestinal cholesterol efflux (TICE) [45-47]. Indeed, several genes related to these mechanisms were shown to be directly regulated by LXR including ABC-transporters (e.g. ABCA1 [48] or ABCG5 [49]) or the HDL receptor SR-BI [20]. The latter has been shown to be modulated by interaction with PDZK1, which we now report to be also directly regulated by LXRα, suggesting that PDZK1 is part of the LXR gene network.

However, our data revealed that PXR represses transcription of PDZK1. In this context, it seems of interest that there are data in the literature pointing to a role of PXR in cholesterol and lipid homeostasis [44, 50]. Indeed, activation of PXR in rodents increased the plasma levels of apoAI and HDL-cholesterol [51], and decreased the mRNA and protein expression of ABCA1 and SR-BI [52]. These findings are in accordance with the observed augmentation of HDL particles size and a lowered SR-BI expression in E3L mice after treatment with the murine PXR agonist (pregnenolone-16α-carbonitrile, PCN) [53, 54]. A similar change in particle size was reported in mice lacking the receptor [55], further supporting the notion that there is a link between PXR, SR-BI and cholesterol homeostasis in rodents, whether this is also true for the human organism cannot be solved at this point.

Further, we also observed the previously described ability of TO 901317 to activate LXRs and PXR [22, 56]. The potential role of TO 901317 as agonist of PXR was investigated by Mitro *et al.* showing that TO 901317 binds to the xenosenor with the same affinity as to LXRα, and showing that the PXR mediated effect was comparable to that observed for the prototypical activator rifampicin [22]. The background for the investigation of the role of PXR in lipid homeostasis were *in vivo* experiments showing different outcomes for GW3965 and TO 901317, although both had been described as LXR agonists. In detail, mice exposed to TO 901317 developed liver steatosis and hypertriglyceridemia [57, 58], while treatment with GW3965 did not lead to pathological changes of triglycerides in liver [59]. The triglyceride accumulation in the liver is explained by the Pxr-mediated upregulation of CD36 (SCARB3), which is a low-density lipoprotein receptor [22, 44].

However, the role of PXR is primarily seen in drug metabolism due to the transcriptional regulation of drug transporters (e.g. OATP1A2 [29] and ABCB1 [60]) and drug metabolizing enzymes (CYP3A4 [61]). In this context, the xenosensor PXR is assumed to balance drug exposure and activity of genes involved in drug metabolism.

In drug metabolism, PDZK1 is linked to drug uptake and efflux transporters (OATP1A2, PEPT2, ABCG2, ABCC2, and ABCC4 [4, 62-65]) suggesting that the scaffold protein modulates transmembrane transport. In detail, PDZK1 stabilizes these transporters at the plasma membrane, which increases their amount in the membrane, and in turn the net transport rate [4, 62-66]. Accordingly, we hypothesize that PXR mediated regulation of PDZK1 influences cellular fluxes, which might be a part of the above-mentioned exposure-elimination balancing function of PXR. However, the overall effect of this regulation, or whether net influx or efflux are more pronounced cannot be answered at this point, and it is beyond the scope of our study to answer this particular question.

Nevertheless, it is remarkable that LXRs and PXR, despite sharing the same half site response element (AG(G/T)TCA), regulate different genes [38, 67]. The *in silico* analysis located the most likely binding site (DR4, direct repeat with 4 base pair spacer, -69 bp to -54 bp) for both nuclear receptors in the 5' deletion fragment of the PDZK1 promoter ranging from -789 bp to +75 bp. However, deletion of this particular DR4 binding site resulted in a significantly reduced transactivation by LXRα, while revealing no change in the repression by PXR. On the one hand, this result suggests that PXR binds to a motif not corresponding to the herein tested DR4, IR1, or IR0 motifs. For the regulation of CYP3A4 it has been shown that PXR binds to ER6 or DR3 motifs [68, 69], but none of them was observed in the 5' deletion PDZK1 promoter fragment. On the other hand, it may suggest that the interaction of PXR with the PDZK1 promoter is of indirect nature. In this context, it seems noteworthy that we recently described regulation of the scaffold protein by the transcription factor hepatocyte nuclear factor 1 homeobox A (HNF1α) [6]. Apart from its auto-

regulation, HNF1α expression is controlled by HNF4α [70]. In 2004, a study showed that rifampicin-activated PXR interferes with the regulation by HNF4α as the nuclear receptor competes for the same cofactor [71]. Preventing the binding of HNF4α to the cofactor resulted in a reduced mRNA amount of target genes such as CYP7A1, which is a key enzyme in cholesterol metabolism [71]. An indirect regulation of the scaffold protein by PXR may therefore be mediated by modulation of HNF4α and indirectly of HNF1α, which is if present constitutively active.

Finally, we investigated the impact of the LXRα and PXR ligands on the mRNA and protein level of PDZK1 and the HDL receptor in HepG2 cells. The LXR agonist GW3965 increased mRNA expression of PDZK1, while incubation with TO 901317 (PXR and LXR ligand) and rifampicin (PXR ligand) did not influence the mRNA amount of *PDZK1* or *SCARB1*. These results are in accordance with the published data of Grefhorst *et al.* where the 14-days treatment with TO 901317 did not change PDZK1 expression in murine liver [21]. On the protein level, we observed a significant decrease in protein expression of PDZK1 for TO 901317, while GW3965 and rifampicin did not significantly affect the expression contrasting the data of Grefhorst *et al.* where the protein expression of Pdzk1 after treatment with TO 901317 did not change. It should be noted that changes in expression were assessed in HepG2 cells, which is certainly a limitation. Even if HepG2 cells are often used to study transcriptional regulation in order to predict behavior *in vivo*, there are reports showing pronounced differences to primary hepatocytes [72, 73]. In addition, PXR is highly variable among species with species specificity in activating ligands [74]. Nevertheless, there are no species differences for TO 901317, which has been shown to activate human, rat, and mouse PXR [74]. However, the exposition of human and rat hepatocytes to GW3965 showed differences in mRNA expression response to lipid homeostasis related genes [75].

Taken together LXRα increased, while PXR repressed the transactivation of PDZK1 promoter in cell-based reporter gene assays. Treatment of HepG2 cells with GW3965 (LXR agonist) resulted in elevated PDZK1 mRNA transcription, but not in a higher protein amount of the scaffold protein in those cells. In contrast, the mRNA expression of PDZK1 remained unchanged after the treatment with TO 901317 (PXR and LXR agonist) while the protein amount was decreased in HepG2. However, the data presented in this study allow the conclusion that the central regulators of the cholesterol/lipid homeostasis (LXRα) and drug metabolism (PXR) are transcriptional regulators of PDZK1.

Conflict of Interest

There is no conflict of interest to be declared by any author.

Authorship

• Conceived of or designed study: Ferreira and Meyer zu Schwabedissen.

• Performed research: Ferreira, Meyer, and Meyer zu Schwabedissen.

• Analyzed data: Ferreira, Meyer, and Meyer zu Schwabedissen.

• Wrote the paper: Ferreira and Meyer zu Schwabedissen.

Figure legends

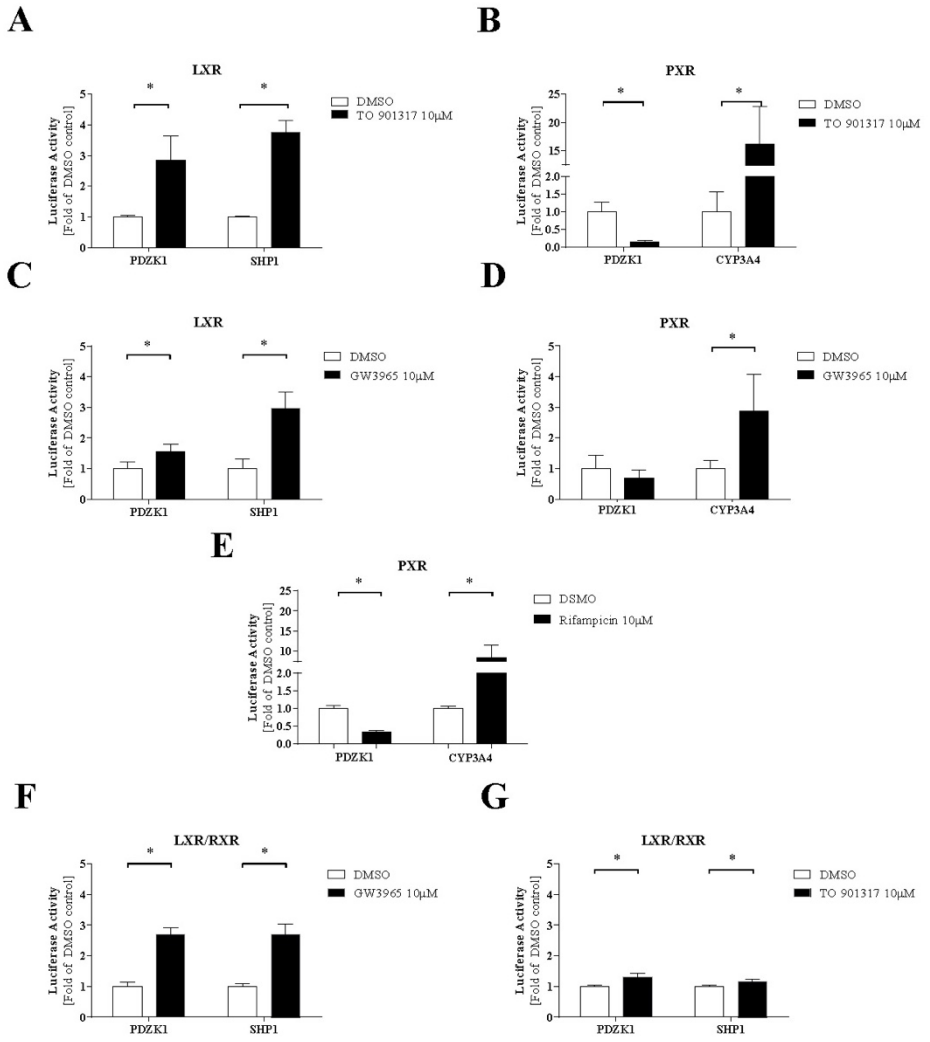

Figure 1 – Comparison of the impact of the specific LXRα agonist GW3965 and the non-specific LXR ligand TO 901317 on PDZK1 transactivation. Dual luciferase reporter gene assays were performed in HepG2 cells transfected with pRL-TK as internal standard, the expression vector pEF6-LXRα or pEF6-PXR, and the luciferase

reporter vectors pGL3-PDZK1promoter, pGL3-SHP1promoter (target of LXRα) or pGL3-XREM-CYP3A4promoter (target of PXR). DMSO was used as control. HepG2 were exposed to TO 901317 (A, B), GW3965 (C, D) and rifampicin (E). Luciferase activity of pGL3-PDZK1promoter and pGL3-SHP1promoter exposed to LXRα and RXRα after treatment with TO 901317 (F) or GW3965 (G). Data are presented as mean ± SD, n = 3 in technical triplicates. For statistical analysis the unpaired Students t- test was used (*$p < 0.05$).

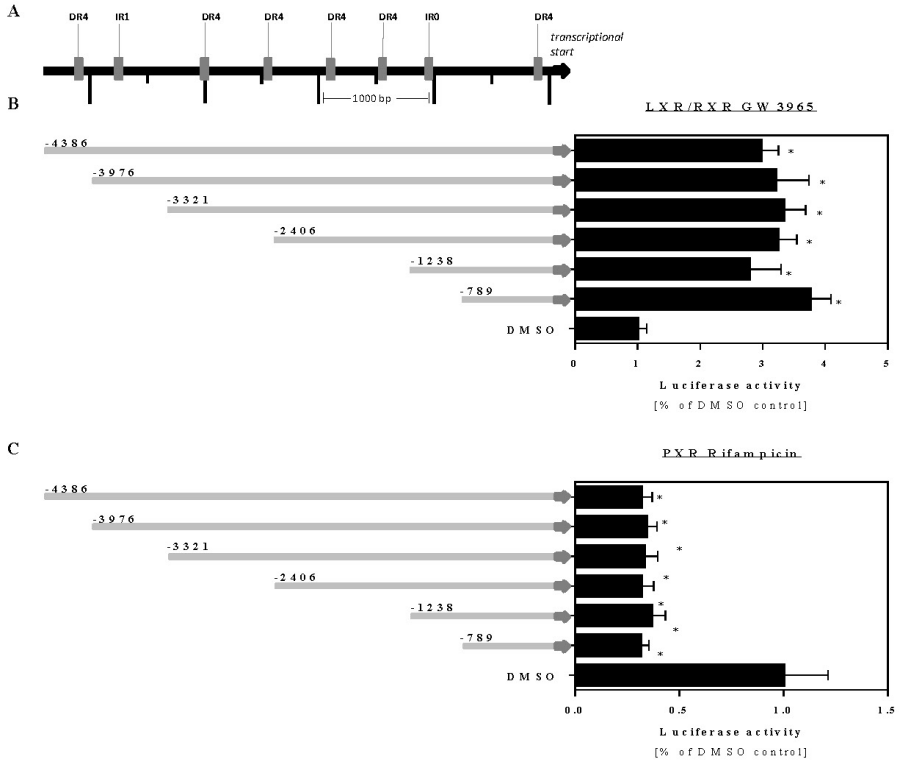

Figure 2- *In silico* analysis of the possible binding site of both nuclear receptors and impact of transactivation in 5' deletion of the PDZK1 promoter. (A) *In silico* analysis of identified potential binding sites by NUBIScan for nuclear receptor LXRα and PXR on the PDZK1 promoter. HepG2 cells were co-transfected with the vectors containing 5'deletion of the PDZK1 promoter and both expression vector for LXRα and RXRα (B) or PXR (C) in presence or

absence of GW3965 or rifampicin, respectively. The PDZK1 promoter 5'deletion constructs of the 4.4 kb promoter of PDZK1 were subcloned into pGL3 basic. Data are presented as mean ± SD (n = 3 in triplicates). For statistical analysis the One-Way ANOVA followed by Dunnett's multiple comparison test was used (*$p \leq 0.05$).

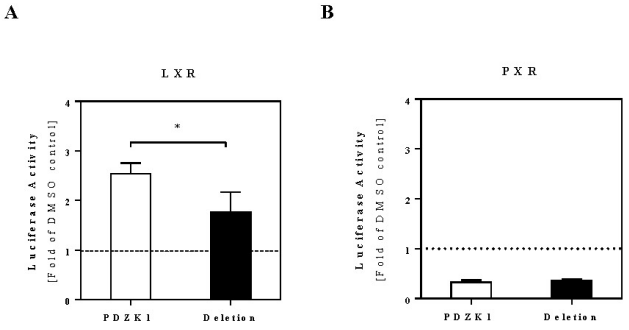

Figure 3: Impact of deletion of the most likely binding site for LXRα and PXR in the PDZK1 promoter. Comparison of the luciferase activity between PDZK1 promoter and deletion of DR4 binding motif for LXRα (A) and PXR (B) after treatment with GW3965 or rifampicin, respectively. Data are presented as mean ± SD, n = 3 in technical triplicates. For statistical analysis the unpaired Students t-test was used (*$p < 0.05$).

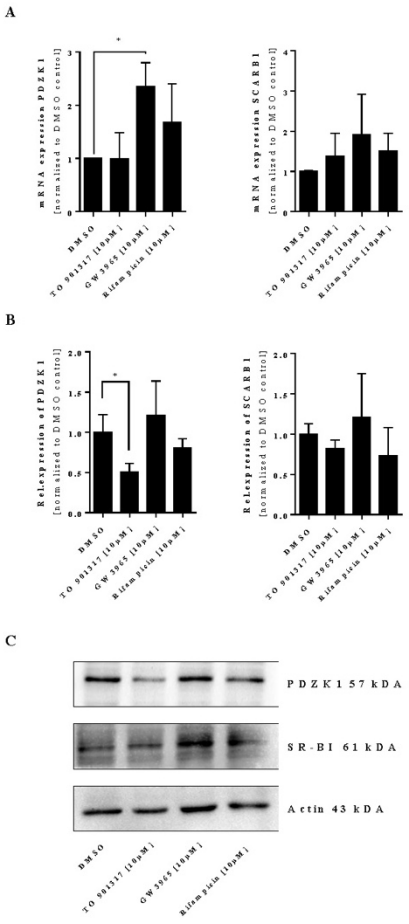

Figure 4: mRNA and protein expression of PDZK1 and *SR-BI* after exposing HepG2 to LXR and PXR-agonist. Change in (A) mRNA expression and (B) protein expression of PDZK1 and SCARB1 after treatment with 10 µM TO 901317, 10 µM GW3965 or 10 µM rifampicin. Data are presented as mean ± SD ((A) n = 6 and (B) n=3 in technical triplicates). For statistical analysis of the mRNA expression the Dunn's multiple comparisons test and for the protein expression one-way ANOVA were used (*$p \leq 0.05$). (C) Protein expression of PDZK1 and SR-BI after treatment was analyzed by immunoblotting. The figure shows one representative of three replications.

Table 1: List of *in silico* identified potential binding sites for LXR and PXR in the PDZK1 promoter. All binding sites were listed according to the position in the PDZK1 promoter (Start = 1). For analysis, the threshold was set at a score of 0.6. The investigated DR4 motif is highlighted in grey.

Repeat	Position	Site sequence
DR4	-4127	CAGTCAtgtgGGGCCA
IR1	-3740	AGGGCAtTGGCCA
DR4	-3015	CAGTCAcaaaAGGACA
DR4	-2436	TGGGCAcggtAGCTCA
DR4	-1877	GGATCActtgAGGTCA
DR4	-1442	GGGTCTcgctCTGTCA
IR0	-1033	AGGTCATGTCTT
DR4	-69	AGGTCActtgAATTCA

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3.3 PDZ domain containing protein 1 (PDZK1), a modulator of membrane proteins, is regulated by the nuclear receptor THRβ

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PDZ domain containing protein 1 (PDZK1), a modulator of membrane proteins, is regulated by the nuclear receptor THRβ

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ABSTRACT

Genome wide association studies revealed single nucleotide polymorphisms (SNP) located within the promoter of PDZ domain containing protein 1 (PDZK1) to be associated with serum uric acid levels. Since modulation of transporters and particularly of membrane proteins involved in uric acid handling by PDZK1 has previously been reported, the aim of this study was to analyze the impact of the polymorphisms rs1967017, rs1471633, and rs12129861 on promoter activity and thereby transcription of PDZK1. Cell-based reporter gene assays showed transactivation of the PDZK1-promoter by triiodothyronine mediated by thyroid hormone receptors (THR) α and β. In silico analysis verified localization of the polymorphism rs1967017 within the most likely THR binding site whose deletion reduced THR-mediated transactivation. Furthermore, our study shows regulation of PDZK1 by thyroid hormones, thereby providing a mechanistic basis for the previously reported associations between thyroid hormone status and uric acid homeostasis.

1. Introduction

Genome wide association studies searching for genetic variants associated with uric acid levels, hyperuricemia, and/or gout in European and Asian populations identified polymorphisms localized in the 5′ untranslated region (5' UTR) of PDZK1 to be linked with the tested phenotypes (Yang et al., 2014,Kolz et al., 2009,Kottgen et al., 2013,van der Harst et al., 2010,Yang et al., 2010). Even if the polymorphisms in this region not always reached the level of genome wide significance (Karns et al., 2012,Dehghan et al., 2008), the observation that PDZK1 is genetically linked to urate homeostasis was basis for further studies finally providing a mechanistic explanation.

PDZK1, a multidomain protein containing four postsynaptic density 95/disk-large/ZO-1 (PDZ)-domains (Kato et al., 2006), functions as scaffold for membrane proteins, thereby contributing to the organization of specialized membrane domains. Indeed, PDZK1 stabilizes multiprotein complexes by direct protein-protein interaction, thus positioning interacting membrane proteins in correct spatial arrangement (Bezprozvanny and Maximov, 2001). Membrane proteins containing the classical type I PDZ-domain binding motif at the carboxy-terminus are likely to directly interact with the PDZ-domains of PDZK1, thereby being stabilized in the membrane and/or modulated in their function (Kato et al., 2006).

In terms of uric acid handling, PDZK1 is assumed to be a stabilizer of the so-called "urate transportosome" (Anzai et al., 2012). Based on our current understanding, the "urate transportosome" is a complex of multiple membrane transporters which contribute to the renal handling of urate. Importantly, PDZK1 interacts with membrane

Abbreviations: 5-HT_2A, Serotonin 2A receptor; ABC, ATP-binding cassette transporter; AhR, Transcription factor aryl hydrocarbon receptor; BCRP, Breast cancer resistance protein; CFTR, Cystic fibrosis transmembrane conductance regulator; DR1, Direct repeat with 1 bp spacer; ERα, Estrogen receptor α; FXRα, Farnesoid X receptor; GRα, Glucocorticoid receptor α; HDL, High-density lipoprotein receptor; MRP, Multidrug Resistance Proteins; NHE3, Sodium-hydrogen exchanger 3; OAT, Organic anion transporter; OATP, Organic anion transporting polypeptide; PDZ, Postsynaptic density 95/ disk-large/ ZO-1; PDZK1, PDZ domain containing protein in kidney 1; PPARα, Peroxisome proliferator-activated receptor α; PXR, Pregnane X receptor; RARβ, Retinoid acid receptor β; RXRα, Retinoid X receptor α; SLC, Solute carrier; SNP, Single nucleotide polymorphisms; SR-B1, Scavenger receptor class B member; THR, Thyroid hormone receptor; THRE, Thyroid response element; URAT1, Urate Anion Exchanger 1; 5′UTR, 5′ untranslated region; VDR, Vitamin D receptor.

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proteins localized at the luminal side of the renal tubule. Accordingly, transporters located in the apical membrane of the tubular cell like the solute carriers 22A12 (SLC22A12, URAT1), or 22A11 (SLC22A11, OAT4) were identified as interacting partners of PDZK1. This interaction has been shown to increase membrane localization, and thereby transport capacity (Anzai et al., 2004,Miyazaki et al., 2005). In addition to those uptake transporters, the ATP-binding cassette transporter C4 (ABCC4, MRP4), an efflux transporter, has been reported to be part of the "urate transportosome" (Van Aubel et al., 2005), and to interact with PDZK1 (Park et al., 2014). Importantly, ABCC4 is also expressed in other tissues such as liver or intestine and is known to play a role in drug metabolism (Giacomini et al., 2010). The list of drug transporters interacting with PDZK1 is further extended by ABCC2 (MRP2), a well-known efflux mechanism for products of the phase II biotransformation that also interacts with PDZK1 (Kocher et al., 1999). In addition to its renal expression, ABCC2 is expressed in the canalicular membrane of hepatocytes and the apical membrane of enterocytes (Jedlitschky et al., 1997), thereby providing evidence that PDZK1, even if highly expressed in kidney, executes its organizational role also in other tissues with polarized cells. This notion is supported by findings on ABCG2 (BCRP) and PDZK1 in murine intestine (Shimizu et al., 2011).

However, at this point it seems noteworthy that the function of PDZK1 is not limited to uric acid handling or drug metabolism, but also affects epithelial fluid transport by modulation of the positioning and gating of ABCC7 (CFTR) (Bezprozvanny and Maximov, 2001). Furthermore, PDZK1 is assumed to modulate cholesterol metabolism interacting with the HDL-receptor scavenger receptor class B member (SCARB1) (Silver, 2004), or to impact neurotransmitter signaling mediated by interaction with the serotonin 2A receptor (HTR2A) (Walther et al., 2015).

Even if the number of interacting proteins and thereby physiological functions modulated by PDZK1 steadily increases, little is known about the mechanisms underlying the observed association of genetic variants located upstream of the coding region of PDZK1 with urate homeostasis. Nonetheless, the area in which the uric acid associated polymorphisms (rs12129861, rs1471633, and rs1967017) are located comprises the promoter region of the scaffolding protein (Tachibana et al., 2008), thereby potentially influencing transcription and expression. The promoter of the scaffolding protein has previously been described by Tachibana, K. et al. reporting transactivation of PDZK1 by PPARα (Tachibana et al., 2008). However, it is currently unknown whether transactivation is influenced by the above-mentioned naturally occurring polymorphisms.

Accordingly, the aim of the herein reported study was to further analyze the 5′UTR of PDZK1 including the abovementioned genetic variants in our considerations. At first, human kidney samples were assessed for expression of PDZK1, revealing a trend for lower mRNA amount in individuals harboring the minor alleles of the polymorphisms. The observed trend in this descriptive analysis was basis for a subsequent mechanistic study screening the promoter for transactivation by known ligand activated nuclear receptors including the thyroid hormone receptor (THRα, NR1A1 and THRβ, NR1A2). This study demonstrates that THRβ activated by T3 induces the expression of PDZK1 and that this transactivation is influenced by naturally occurring polymorphisms, as observed in the herein reported *in vitro* experiments.

2. Materials and methods

2.1. Kidney tissue samples

Human malignant and adjacent non-malignant kidney samples were obtained from patients undergoing surgery due to kidney tumors after their written informed consent. The study was approved by the local ethics committee of the Medical Faculty of the University of Greifswald (III UV 12/03). Once the tissue samples were prepared and pulverized as reported elsewhere (Prestin et al., 2016), the samples were stored at −80 °C until further use.

2.2. Cell culture

Cell lines were cultured at 37 °C in a humidified 5% CO_2-atmosphere. Dulbecco's Modified Eagle's Medium (D6429, 4.5 mg/L glucose and 1 mM sodium pyruvate, Sigma-Aldrich, Arlesheim, Switzerland) was used as culture medium for HepG2 (ATCC® HB-8065™) and Caco2 (ATCC® HTB-37™), while M199 (Gibco™, LuBioScience, Lucerne, Switzerland) was used for BeWo cells. The medium was supplemented with 5% FCS (AMIMED, BioConcept Ltd., Allschwil, Switzerland), 1% GlutaMAX™, (Thermo Fisher Scientific, Reinach, Switzerland). Caco2 cells were cultivated for 14 days, before starting the experiments. Human primary renal proximal tubule epithelial cells (RPTEC) derived from renal tissue of a Caucasian 55-year old male (Lot 0000324297) were obtained from RUWAG (Bettlach, Switzerland) and cultured in renal epithelial growth medium supplemented with 0.5% serum, 0.1% rhEGF, 0.1% insulin, 0.1% hydrocortisone, 0.1% epinephrine, 0.1% triiodothyronine, and 0.1% transferrin (Clonetics™ REGM™ BulletKit™, Lonza, Basel, Switzerland). Cell passaging of RPTEC was performed using the DetachKit (PromoCell, Heidelberg, Germany) containing HEPES buffered saline solution (30 mM HEPES), trypsin-EDTA solution (0.04% and 0.03%), and trypsin neutralizing solution (0.05% with 0.1% BSA). All cell lines were tested negative for contamination with mycoplasma.

2.3. Genotyping of kidney samples

Genomic DNA from the renal tissue was isolated with the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The renal DNA was used for genotyping of rs1967017 (PDZK1 -4017 bp T > C), rs1471633 (PDZK1 -3923 bp A > C), and rs12129861 (PDZK1 -1976 bp G > A). Analysis was performed using commercially available TaqMan® genotyping assays (C___1862243_10, C___1862244_10, or C__26662675_10, LuBioScience), the ViiA™7 Real-Time PCR System, and the ViiA™7 software v1.2.2 (Thermo Fisher Scientific). However, the assay C___1862244_10 for genotyping of rs1471633 was not specific, as observed by analysis of fluorescence data after amplification according to the manufacturer's instructions. Therefore, the resulting PCR product of each sample was subsequently digested using *Hpy*CH4III (*Taa*I) (Thermo Fisher Scientific) finally identifying the genotype by RFLP. In case of exchange of A to C, the *Hpy*CH4III was not capable of cutting the PCR fragment (supplemental Fig. A1).

2.4. Western blot analysis of human kidney samples and cell lines

Protein expression of cell lines and human kidney samples was analyzed by immunoblotting. Samples were separated by SDS-PAGE and electro-transferred onto a nitrocellulose membrane using a tank blotting system (Mini Trans-Blot® Cell; Bio-Rad Laboratories, Cressier FR, Switzerland). After blocking with 5% FCS diluted in tris-buffered saline supplemented with TWEEN®20 (TBST), the membranes were incubated with the respective primary antibody at 4 °C overnight. For detection of PDZK1 and β-actin, the rabbit monoclonal anti-PDZK1 antibody ab137873 (Lucerna-Chem, Lucerne, Switzerland; diluted 1:2500 in TBST with 1% BSA) and goat polyclonal anti-β-actin antibody sc-1616 (Santa Cruz Biotechnology, Heidelberg, Germany; diluted 1:1000 in TBST 1% BSA) were

used, respectively. Secondary horseradish peroxidase (HRP)-conjugated antibodies (Bio-Rad Laboratories; diluted 1:2000 in TBST 1% BSA) were used to detect the immobilized primary antibody. For visualization of the HRP-conjugated secondary antibody, the Western ECL Substrate (Thermo Fisher Scientific) was used. Chemiluminescence was digitalized with the ChemiDoc™ MP System (Bio-Rad Laboratories) and analyzed with the Image Lab™ Software 4.1 (Bio-Rad Laboratories).

2.5. Real-time PCR

The tissue RNA used for comparison of expression was obtained from AMS Biotechnology (Bioggio-Lugano, Switzerland). For isolation of RNA from cell lines, pegGold RNAPure™ (Axon Lab, Baden-Dättwil, Switzerland) was used as described by the manufacturer. After spectrometric quantification, 1000 ng of RNA were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (LuBioScience). The resulting cDNA was basis for mRNA quantification using the ViiA™7 Real-Time PCR System (Thermo Fisher Scientific). The amount of mRNA of PDZK1, 18S ribosomal RNA (18S rRNA), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and RNA Polymerase II Subunit A (POLR2A) was quantified using the commercially available TaqMan® gene expression assays (LuBioScience Hs00275727_m1, Hs4319413E, Hs4310884E, and Hs01108274_m1, respectively). CT-values observed for the reference genes were analyzed using the open access software RefFinder, suggesting POLR2A as the most stable gene of reference in our data set. Data were analyzed by the 2^-ΔΔCT method, where CT values of the gene of interest were normalized to that of POLR2A detected in the same sample (ΔCT). The ΔCT values of each sample were referred to the mean ΔCT value of the indicated control, resulting in the ΔΔCT.

2.6. In silico scan for transcription factor binding motifs

In silico screening of the promoter of PDZK1 (NM_002614.4) for potential binding sites of several nuclear receptors was performed with the open source program PROMO (ver.3.0.2) which is based on the algorithms of Transfac (ver.8.3) (Messeguer et al., 2002,Farre et al., 2003). Subsequent calculations for the THRβ binding sites were conducted with the open access program NUBIScan version 2.0 (www.nubiscan.unibas.ch). The underlying algorithm is a joining of weighted distribution matrices of nucleotide hexamer half sites as published by Podvinec et al. (2002). The scanned sequence was a fragment comprising base pairs −4386 to +75 of the 5′UTR of the gene encoding for PDZK1 (NM_002614.4). The search parameters were set on automatic, while the threshold was set to 0.7. The search matrix was generated based on previously described specific binding pattern for THRβ (Ayers et al., 2014).

2.7. Cloning of PDZK1 promoter fragments

A 4461 bp fragment (−4386 bp to +75 bp) of the 5′UTR of the PDZK1 gene was amplified using the following primer pair PDZK1prom_for 5′-TTGGTACCCTCCAAGAGTGCCAAGACCAGAC-'3 and PDZK1prom_rev 5′-TTCTCGAGGAAGAGGAGCTGCTCTGTTCGTTC-'3. After digest with the FastDigest enzymes *Kpn*I and *Xho*I (Thermo Fisher Scientific), the fragment was ligated into the multiple cloning site of pGL3-Basic (Promega, Dübendorf, Switzerland). After amplification in *Escherischia coli* and control by Sanger sequencing (Microsynth, Balgach, Switzerland), the plasmid was template for the cloning of shorter 5'deletion fragments starting at −2406 bp using *Hin*dIII, at −1238 bp using *Sac*I and *Xho*I, and at −789 bp using *Nhe*I and *Hin*dIII. The fragments comprising the base pairs −3976 to +75, and −3679 to +75 of the PDZK1 promoter were amplified by PCR using the primers 3976_forward 5′-GGTACCGTCGCCTCTTAAGGT-'3 and 3976_reverse 5′-CCATCTTCCAGCGGATAG-'3, and −3679_forward 5′-GGTACCGGGCTTTGGCTAAGTC′-3 and −3679_reverse 5′-AGGAACCAGGGCGTATCT′-3, respectively. The fragments were ligated in the multiple cloning site of pGL3-Basic after digestion with *Kpn*I and *Xho*I. Naturally occurring genetic variants including the single nucleotide polymorphisms rs19607017 (allele T or C), rs1471633 (allele C or A), and rs12129861 (allele G or A) were introduced into the −4386 bp to 75 bp PDZK1 promoter fragment using the primers summarized in supplemental Table A1 and the QuikChange™ Multi Site-Directed Mutagenesis Kit from Agilent Technologies (Basel, Switzerland) according to the manufacturer's protocol.

2.8. Generation of promoter fragments containing mutation of the predicted binding sites

The potential binding site for the THRβ identified by *in silico* analysis and located in the region of maximal activation in the reporter gene assay was deleted performing an overlap extension PCR. Briefly, the −4386 bp to +75 bp fragment containing plasmid served as DNA template in two PCRs using the mutation primers DR1_rev in the first PCR and DR1_for in the second PCR. Both primers were complementary and comprised the sequence flanking the site to be deleted.

For amplification of the region from −4386 bp to −4023 bp the following primers were used RV3 5′-CTAGCAAAATAGGCTGTCCC-3′ and DR1_rev 5′-GGAGGAATTTATTAGGCCA GGATGCAAGACTGAGA-TAAAT-3'. In addition, the region from −4012 bp to +75 bp was amplified using the primers GL2 5′-CTTTATG TTTTTGGCGTCTTCC-3′ and DR1_for 5′-ATTTATCTCAGTCTTG-CATCCTGGCCTAATAAATTCCTCC-3'. The products resulting from the two PCRs were then gel purified and simultaneously applied to a third PCR using the primer GL2 and RV3 amplifying the fragment -4386 bp to +75 bp with the deleted binding site. The resulting PCR-fragment lacking the DR1 motif was subcloned into pGL3-Basic using the restriction enzymes *Kpn*I and *Xho*I, transformed in bacteria for amplification, and finally controlled by sequencing (Microsynth).

2.9. Cell-based reporter gene assays

The pEF6/V5-His-plasmids containing the coding sequence of nuclear receptors PXR (NR1I2), FXRα (NR1H4), THRα (NR1A1), THRβ (NR1A2), and PPARα (NR1C1) have previously been described (Meyer zu Schwabedissen et al., 2008, Meyer zu Schwabedissen et al., 2010). However, the preparation of the plasmids is briefly summarized in supplemental Table A2. The impact of nuclear receptors on transactivation of PDZK1 was assessed performing cell-based reporter gene assays. In detail, prior to transfection using 3 μl jetPRIME® transfection reagent (Polyplus, Chemie Brunschwig, Basel, Switzerland) per microgram DNA, HepG2 cells were seeded in 24-well plates at a density of 80,000/well. In total, 25 ng pRL-TK (Promega) encoding for *Renilla* luciferase, 250 ng of the respective pEF6 expression vector, and 250 ng of the respective PDZK1 promoter construct in pGL3-Basic were used for transient transfection per well. The day after transfection cells were treated with the respective activating ligand. Rifampin, chenodeoxycholic acid (CDCA), fenofibrate, or 3, 3′, 5-triiodo-L-thyronine (T3) (each obtained from Sigma-Aldrich) were used for activation of PXRα, FXRα, PPARα, THRα, or THRβ, respectively. Each compound was prepared as a 1000 × DMSO stock solution of the indicated concentration. A concentration of 0.1% DMSO served as solvent control. Prior to quantification of *Renilla* and luciferase activity with the Dual-

Luciferase® Reporter Assay System (Promega), the cells were lysed in 120 µl lysis buffer (Promega). Finally luciferase activities were quantified in 20 µl of the lysate with the Infinite® M200 Pro (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions. Activity of firefly luciferase was normalized to that of *Renilla*. Transactivation by thyroid hormones is reported as fold of DMSO control. Each plasmid served as its control to avoid the effect of changes in transfection efficiency due to plasmid size.

2.10. Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling, Allschwil, Switzerland) following the manufacturer's protocol. Briefly, Caco2 cells were cultivated for 14 days and then treated 48 h with 10 µM T3 or DMSO before starting the ChIP assay. We used 5 µg digested, crosslinked chromatin for each reaction. For detection of THRβ binding, one sample was incubated with rabbit polyclonal anti-THRβ antibody ab180612 (Lucerna-Chem; 200 ng/µl) at 4 C° overnight. The PCR was performed with the HotStarTaq® DNA polymerase (QIAGEN, Hombrechtikon, Switzerland), the sense primer 5′-CTCCAAGGATCCTCCCCTGTCCTGACA-3′, and the antisense primer 5′-GACAGTCTCCCTTTCCAC-3′ generating an amplicon of 270 bp encompassing the *in silico* identified DR1 motif. The resulting PCR product was verified on a 2% agarose gel with a 100 bp GeneRuler™ DNA ladder (Thermo Fisher Scientific). For negative control, crosslinked chromatin was incubated with normal rabbit IgG (Cell Signaling). For positive control crosslinked chromatin was incubated with anti-Histone H3 (D2B12) XP® Rabbit antibody (Cell Signaling), and PCR was performed using DNA primers provided in the assay by the manufacturer (Cell Signaling).

2.11. Statistical analysis

Data are presented as mean ± standard deviation (SD). Data analysis was performed using the GraphPad Prim software 6.04 (GraphPad Software, San Diego CA, U.S.A.) and Microsoft Excel (Microsoft, Redmond WA, U.S.A.). $P \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Influence of genetic variants on expression of PDZK1 in human kidney samples

At first we tested whether the polymorphisms rs1967017, rs1471633, and rs12129861 located in the 5′UTR of PDZK1 influence the expression of the scaffold protein in human kidney (Fig. 1). Even if we did not observe statistically significant differences, there was a trend of lower PDZK1 mRNA-levels in kidney of humans homozygous for the minor alleles of the tested polymorphisms. It seems noteworthy that two polymorphisms (rs1967017 and rs1471633) were linked in the tested population. At the same time we determined protein expression and tested whether the amount of protein detected in the renal samples was influenced by the genetic variants. As shown in Fig. 1 there was no statistically significant effect. However, due to the low number of individuals ($n = 27$) summarized in our tissue collection, we considered the consistent trend of lower mRNA as sufficient evidence to further evaluate the impact of genetic variants on transcriptional activity of the gene.

3.2. Basal promoter activity comparing the different genetic variants

To determine whether the polymorphisms located in the promoter region of PDZK1 influence basal promoter activity, promoter fragments containing all possible nucleotide combinations of the three polymorphisms rs19670171, rs1471633, and rs12129861 were tested in cell-based reporter gene assays. Assessment of basal promoter activity in HepG2 cells revealed significantly enhanced luciferase activity in all cells transfected with the promoter fragments compared to those transfected with the pGL3-Basic vector control. However, the observed basal promoter activity did not differ significantly comparing all possible haplotypes (Fig. 2).

3.3. In silico analysis of the 4.4 kb 5′untranslated region of PDZK1

The *in silico* analysis of the PDZK1 promoter fragment using the open source program PROMO (ver.3.0.2) revealed a variety of potential binding sites for ligand-activated nuclear receptors including the vitamin D receptor (VDR, NR1I1), the retinoid X receptor α (RXRα, NR1B1), the retinoid acid receptor β (RARβ, NR1B2), the estrogen receptor α (ERα, NR3A1), the glucocorticoid receptor α (GRα, NR3C1), the peroxisome proliferator-activated receptor α (PPARα, NR1C1), the thyroid hormone receptor β (THRβ, NR1A2), and the transcription factor aryl hydrocarbon receptor (AhR) as illustrated in supplemental Fig. A2.

3.4. Screening of nuclear receptors for transactivation of PDZK1

PDZK1 has been shown to interact with a variety of drug transporters including the efflux transporters ABCC2 (MRP2), and ABCC4 (MRP4) and the uptake transporter OATP1A2 (OATP-A) (Park et al., 2014,Kocher et al., 1999,Zheng et al., 2014). It is known that nuclear receptors such as THRα and THRβ (Fujiwara et al., 2001), the farnesoid X receptor (FXRα) (Meyer zu Schwabedissen et al., 2010), and the pregnane X receptor (PXRα) (Meyer zu Schwabedissen et al., 2008) are involved in their regulation and/or that some of the activating ligands are substrates of the abovementioned transporters. To test whether these ligand-activated nuclear receptors modulate expression of PDZK1, cell-based reporter gene assays were performed. In addition, the nuclear receptor PPARα was included in the screening, as this particular transcription factor has previously been reported to modulate transcription of the scaffolding protein (Tachibana et al., 2008). Determination of luciferase activity revealed significantly enhanced PDZK1 promoter activity in presence of ligand activated PPARα (mean luciferase activity ± SD; DMSO vs. 30 µM fenofibrate: 1.000 ± 0.008 vs. 1.638 ± 0.147; $P = 0.003$), THRα (DMSO vs. 10 µM T3: 1.000 ± 0.0914 vs. 8.482 ± 1.252; $P = 0.005$), and THRβ (DMSO vs. 10 µM T3: 1.000 ± 0.081 vs. 7.190 ± 0.695; $P = 0.001$) (Fig. 3). No enhanced transactivation was observed for FXRα the sensor of bile acids (DMSO vs. 50 µM CDCA: 1.000 ± 0.391 vs. 0.614 ± 0.181; $P = 0.197$, data not shown). However, the xenosensor PXR significantly decreased luciferase activity (DMSO vs. 10 µM rifampin: 1.000 ± 0.089 vs. 0.328 ± 0.043; $P = 0.003$) (data not shown).

3.5. Identification and verification of potential binding sites for THRβ

Our primary screening of various ligand-activated nuclear receptors revealed significantly enhanced luciferase activity in cells treated with triiodothyronine (T3) in presence of the heterologous thyroid receptors THRα or THRβ. Due to the fact that the latter is the predominant isoform of the THRs in highly metabolic active tissues such as kidney and liver, where PDZK1 has been reported to be expressed, we focused our further investigation on THRβ (Williams, 2000). A NR-defined *in silico* analysis of the −4386 bp to +75 bp fragment of the 5′untranslated region of the PDZK1 gene was performed using the open access software NUBIScan to further

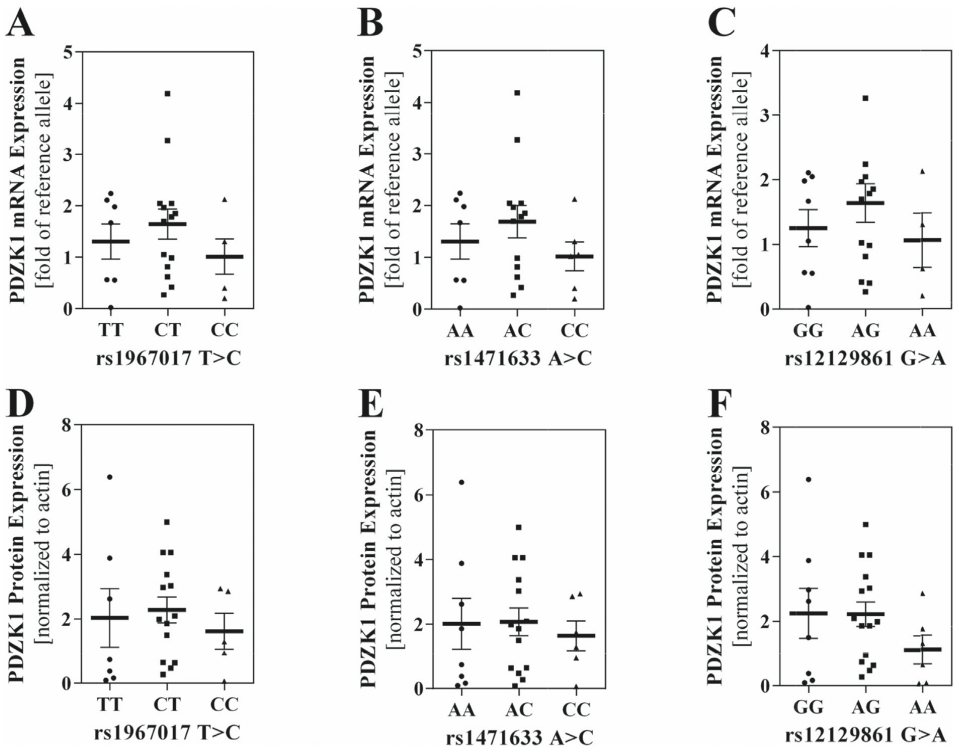

Fig. 1. Impact of genetic variants on PDZK1 mRNA and protein expression in human kidney samples ($n = 27$). A, B, C: The amount of PDZK1 mRNA was determined by real-time PCR and normalized to that of POLR2A. Subsequently, the normalized expression related to the expression observed in samples harboring the reference nucleotide was used for analysis of the impact of the genetic variants rs1967017 (PDZK1 -4017 bp T > C) rs1471633 (PDZK1 -3923 bp A > C), and rs12129861 (PDZK1 -1976 bp G > A). D, E, F: Protein expression was determined by Western blot analysis. Densitometrically quantified PDZK1 protein expression was normalized to that of β-actin and analyzed for the influence of the above mentioned genetic variants. Data are presented as mean ± S.E.M. For statistical analysis the One-Way ANOVA followed by Dunnett's multiple comparison test was used ($P \leq 0.05$ would have been considered statistically significant).

support the notion of THRβ being involved in the regulation of the PDZK1 promoter. This analysis revealed a variety of potential THRβ binding sites as summarized in supplemental Table A3. Subsequently several 5′-deletion fragments of the PDZK1 promoter were generated and tested for transactivation by ligand activated THRβ *in vitro*. As shown in Fig. 4A, luciferase activity was significantly increased in presence of the −4386 bp to +75 bp, and the −3976 bp to +75 bp fragment. However, the latter exhibited much lower activity, indicating that the major THRβ response element is most likely located between positions −4386 bp and −3976 bp. Based on our *in silico* analysis, there are two potential thyroid hormone response elements (TRE), a direct repeat with 4 bp spacer (DR4) (AGGCCTccccTGTCCT) in position −4179 bp to −4164 bp and a direct repeat with 1 bp spacer (DR1) (TGGACTcTGGCCT) in position −4022 bp to −4010 bp (Fig. 4A).

3.6. Impact of deletion of the putative THR binding site on transactivation of PDZK1

Considering the finding from the *in silico* analysis which revealed a DR1 motif for THRβ between −4022 bp to −4010 bp and the fact that the polymorphism rs1967017 is located within this motif at −4017 bp, we further evaluated the role of the DR1 motif in the transactivation of the promoter. Deletion of the DR1 motif resulted in a significantly diminished transactivation of the promoter by THRβ (mean percentage of the wildtype ± SD; wildtype vs. mutated DR1 site: 100.00% ± 6.88% vs. 56.23% ± 9.02%; $P = 0.0058$; Fig. 4B).

3.7. Influence of naturally occurring genetic variants on transactivation of PDZK1 by THRβ

Testing the impact of the naturally occurring single nucleotide exchanges in the 5′UTR of PDZK1 on transactivation we observed an influence of rs1967017. In detail, comparing the activity of the wildtype promoter fragment named "TAG" containing the nucleotide combination T for rs1967017, A for rs1471633, and G for rs12129861 (mean luciferase activity ± SD: 1.000 ± 0.122) to all other possible haplotypes, we observed a significant lower THRβ-mediated transactivation of the fragments CCG (mean luciferase activity normalized to TAG: 0.62 ± 0.160), CAA (0.70 ± 0.151), or CCA (0.69 ± 0.131), thereby suggesting that transactivation is influenced by the minor allele of rs1967017. However, there was only a trend towards reduced transactivation for CAG (0.78 ± 0.176). The activity of fragment TAG did not differ from that of the variants TCA (0.98 ± 0.170), TAA (1.01 ± 0.097), or TCG (1.099 ± 0.266) (Fig. 4C).

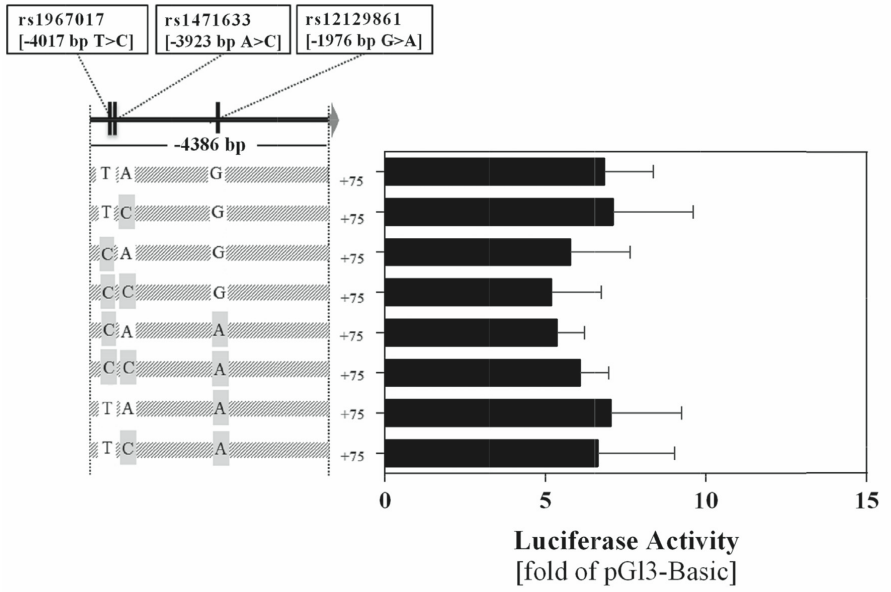

Fig. 2. Influence of genetic variants on the basal promoter activity of PDZK1. Basal promoter activity was assessed in cell-based reporter gene assays comparing luciferase activity after transfection of HepG2 cells with plasmids containing the −4386 bp to +75 bp fragment of the PDZK1 promoter where the minor allele of the polymorphisms rs1967017, rs1471633, and rs12129861 was introduced by site-directed mutagenesis. Data are presented as mean ± SD ($n = 3$ in triplicates). For statistical analysis the One-Way ANOVA followed by a post-hoc Bonferroni test was used. A P-value ≤ 0.05 would have been considered statistically significant.

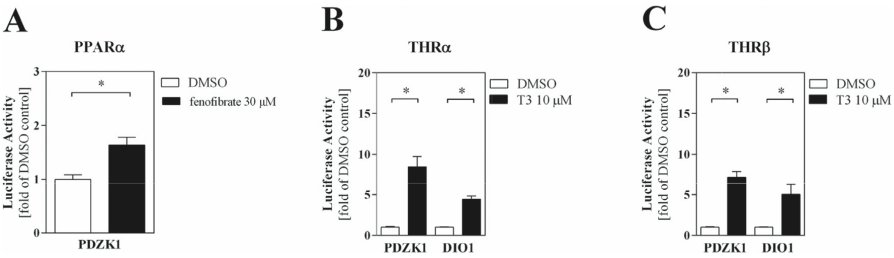

Fig. 3. Screening of nuclear receptors for transactivation of PDZK1 in cell based reporter gene assays. Luciferase activity was assessed after transfection of HepG2 cells with the indicated reporter gene construct and with the expression vector of the indicated nuclear receptor (NR). Cells were treated with the respective activating ligand. A: The NR peroxisome proliferator-activated receptor alpha (PPARα, NR1C1) and its activating ligand fenofibrate were used to verify transactivation of the PDZK1 promoter. B, C: Thyroid hormone receptors alpha (THRα, NR1A1) and beta (THRβ, NR1A2) were activated with triiodothyronine (T3). The promoter of the THR target gene type I iodothyronine deiodinase promoter (DIO1) served as control. Data are presented as mean ± SD ($n = 3$ in triplicates). For statistical analysis the non-paired Student's t-test was used (*$P \leq 0.05$).

As mentioned before, the polymorphism rs1967017 was linked to rs1471633, accordingly the haplotypes CCG or CCA are expected to occur in a population.

3.8. Screening of tissues and cell lines to determine presence of PDZK1 and THRβ

To select a cellular model for validation of the regulation of PDZK1 by the thyroid hormone receptor, we quantified mRNA expression of PDZK1 and THRβ in different human tissues and commonly used model cell lines. All investigated tissues showed an expression of THRβ (relative expression normalized to liver; intestine: 1.133; kidney: 3.169; liver: 1.000 and placenta: 3.578), whereas PDZK1 was only expressed in intestine (4.429), kidney (33.661), and with a lower amount in liver (1.000) (data not shown). Similar results were obtained in the cell lines. For further experiments we selected Caco2 cells, since they exhibited the most pronounced expression of both genes (PDZK1 6.323 and THRβ 4.171), compared to the other tested cell models (RPTEC: 1.891 and 2.032, HepG2: 2.044 and 1.111, BeWo: 0.135 and 0.738; Fig. 5A and B).

3.9. Validation of the impact of T3 on PDZK1 expression in Caco2 cells

Even if it is known that THRα is the predominant thyroid

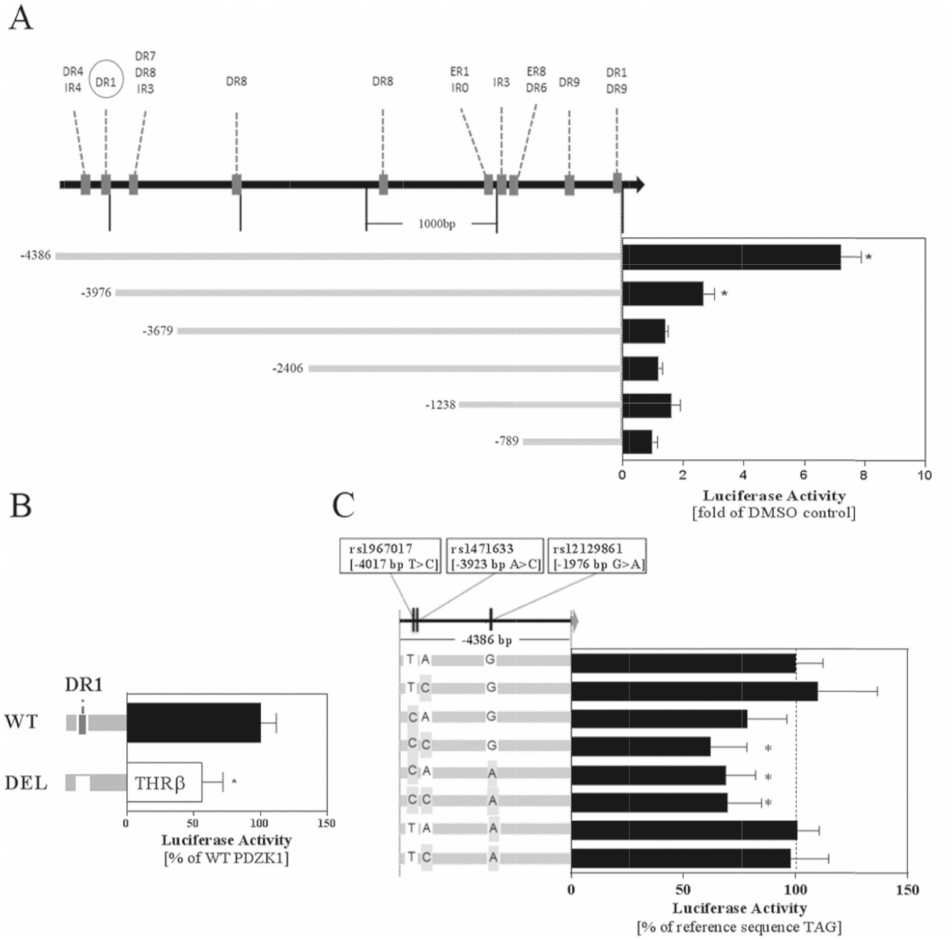

Fig. 4. Detection of the most likely THRβ binding motif in the PDZK1 promoter and impact of the polymorphisms on transactivation. Cell-based reporter gene assays were performed in transfected HepG2 cells. Luciferase activity was assessed after treatment with 10 μM triiodothyronine (T3) in presence of heterologous THRβ. A: Luciferase activity in presence of 5′-truncated fragments of the 4.4 kb promoter of PDZK1. The scheme indicates the localization of the *in silico* predicted THRβ binding motif, where the direct repeats DR1 is highlighted by circles ($n = 3$ in triplicates). B: Reporter gene activity in presence (WT) and absence (DEL) of the DR1 motif ($n = 3$ in triplicates). C: Impact of naturally occurring genetic variants on transactivation of the PDZK1 promoter. Luciferase activity was normalized to that observed for the plasmid with the reference sequence T for rs1967017, A for rs1471633, and G for rs12129861 (haplotype TAG) ($n = 5$ in triplicates). Data are presented as mean ± SD. For statistical analysis the One-Way ANOVA followed by Dunnett's multiple comparison test was used (*$P \leq 0.05$).

hormone receptor isoform in Caco2 cells (Malo et al., 2004) we tested whether THRβ is capable of binding to the herein identified DR1 motif of the PDZK1 promoter performing a ChIP assay. The PCR performed using primers specific for the region flanking the identified THRβ response element in position −4022 bp to −4010 bp of PDZK1 promoter resulted in a 269 bp PCR product when an antibody directed against THRβ was used to enrich genomic DNA from T3 treated cells. This confirmed binding of this nuclear receptor to this particular region of the promoter. In addition, a PCR amplicon of the same size was detected when non-immunoprecipitated chromatin was used as input control. No product was observed in the negative control using rabbit IgG for detection of unspecific binding. An amplicon of 161 bp was detected in the positive control after immunoprecipitation with an antibody directed against histone H3 and using primer restricting exon 3 of ribosomal protein L30 (RPL30) which is known to be associated to the protein histone H3 (Fig. 5 C).

To test whether THR ligands modulate expression of PDZK1 in enterocytes, Caco2 cells were treated with both T3 and T4 for 48 h. Treatment with both hormones significantly increased mRNA expression of the scaffolding protein (mean PDZK1 mRNA-expression normalized to DMSO control ± SD: 1.000 ± 0.125; T3: 2.891 ± 0.865, $P = 0.0286$; T4 2.067 ± 0.712, $P = 0.0286$; Fig. 5D and E). Similar results were obtained analyzing the impact of T3 and T4 on the protein expression of PDZK1 (mean PDZK1 expression normalized to β-actin ± SD compared to DMSO control:

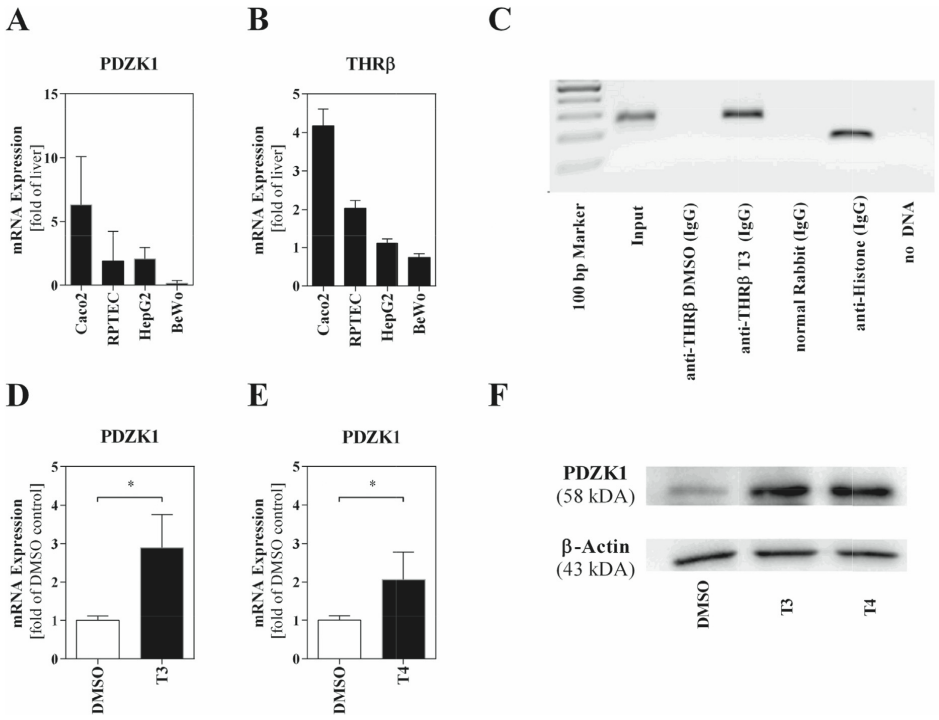

Fig. 5. Validation of the impact of THRβ on PDZK1 expression in Caco2 cells. A–B: Endogenous expression of PDZK1 and THRβ in different human cell types. Data are normalized to expression in liver ($n = 3$ in technical duplicates). C: Validation of the THRβ binding to the PDZK1 promoter. Caco2 cell were cultivated for 14 days and then treated with 10 μM T3 or DMSO for 48 h. The figure shows the gel-separated PCR products of the chromatin immunoprecipitation (ChIP) assay using nuclear extract of Caco2 cells. For input control non-immunoprecipitated chromatin was used (2% Input). Immunoprecipitation of crosslinked chromatin with rabbit anti-THRβ antibody revealed the binding of THRβ in the genomic region containing the DR1 motif (anti-THRβ T3 (IgG)) located in the PDZK1 promoter. For negative control normal rabbit IgG was used for detection of unspecific binding of proteins, while the histone H3 (D2B12) XP® rabbit antibody and DNA primers encompassing the exon 3 of human RPL30 provided by the manufacturer served as positive control (anti-Histone (IgG)). D–E: The mRNA expression of PDZK1 after treatment with T3 or T4 was assessed by real-time PCR. Data are presented as mean ± SD ($n = 4$ in triplicates). For statistical analysis the Mann-Whitney test was used (*$P \leq 0.05$). F: Protein expression of PDZK1 after treatment with T3 or T4 was analyzed by immunoblotting and normalized to β-actin. The figure shows one representative of three replications.

1.000 ± 0.335; T3: 2.956 ± 0.923, $P = 0.0260$; T4: 3.503 ± 1.343, $P = 0.0351$; Fig. 5 F).

4. Discussion

In this study we provide evidence for the regulation of the scaffolding protein PDZK1 by the thyroid hormone receptor β (THRβ), as we observed transactivation of the PDZK1 promoter in cell-based reporter gene assays in presence of this nuclear receptor and its activating ligand triiodothyronine (T3). Additionally, a transactivation by thyroid hormone receptor α (THRα) was observed. The transactivation by THRβ was significantly diminished after mutation of the *in silico* identified TRE located in position −4022 bp to −4009 bp, and was modified by presence of the polymorphism rs1967017. Finally, mRNA and protein expression of the scaffold protein were significantly enhanced in T3-or T4-treated Caco2 cells, supporting the idea of THRs being regulators of PDZK1 and suggesting that there is a link between thyroid hormone status and PDZK1 expression.

Since PDZK1 is a modulator known to influence localization and function of membrane proteins (Walsh et al., 2015), alterations in expression of this scaffold protein could be one mechanism contributing to interindividual differences in membrane protein function.

Among the membrane proteins, a wide range of solute carriers (SLC) and ATP binding-cassette (ABC) transporters has been reported to interact with PDZK1, thereby being modulated in function. Our results showing regulation of PDZK1 by thyroid hormones suggest, that thyroid hormone status would influence activity of those transporters. Particularly the SLC- and ABC-transporter located at the apical pole of renal tubular cells, which are summarized in the so called "urate transportosome" have been extensively studied for their modulation by PDZK1 (Anzai et al., 2012). The influence on tubular handling of the endogenous metabolite of purine metabolism balancing uptake and efflux is exemplified by the interaction of PDZK1 with the uric acid uptake transporter SLC22A12 (URAT1) (Anzai et al., 2004) and the efflux transporter ABCC4 (MRP4) (Park et al., 2014), both known to be expressed at the luminal pole of tubular cells.

The impact of an altered thyroid hormone status on kidney function in uric acid homeostasis has been focus of several studies. Even if most of these studies investigated the influence of renal dysfunction on the endocrine system (Montenegro et al., 1996,Nakahama et al., 2001,Lafayette et al., 1994,Ejaz et al., 2007),

a few of them focused on the influence of thyroid hormone status on uric acid levels, thereby revealing that both overt hypothyroidism and hyperthyroidism are linked with increased serum uric acid levels (SUA) (Kuzell et al., 1955,Leeper et al., 1960,Mooraki and Bastani, 1998,Zhang et al., 2016,Ye et al., 2015). However, various underlying mechanisms are discussed, since uric acid plasma level is the result of multiple renal functions including glomerular filtration, tubular reabsorption, and secretion as well as altered synthesis of this product of purine metabolism. Regarding glomerular filtration Giordano et al. suggested that hyperuricemia in patients with hypothyroidism is secondary due to decreased renal plasma flow, which would certainly impair glomerular filtration and thereby uric acid elimination (Giordano et al., 2001). Accordingly, it has been shown that treatment of hypothyroidism caused enhanced urate diuresis, thereby reducing SUA levels in patients (Arora et al., 2009,Chou et al., 2011). Even though, as the nephron has to be considered as functional unit, it is impossible to dissect whether glomerular filtration or tubular handling is the major driving force of serum uric acid changes during hypothyroidism. However, hyperthyroidism has also been reported to be linked with elevated SUA levels, which are normalized by therapy with anti-thyroid agents (Ford et al., 1989). In contrast to the hypothyroid hyperuricemia, elevated serum uric acid levels associated with hyperthyroidism are assumed to be the result of an accelerated uric acid production due to increased purine metabolism (Giordano et al., 2001,Ford et al., 1989,Sato et al., 1995). Nonetheless, the data from literature indicate that SUA and thyroid hormones are connected, and our findings showing thyroid hormone-mediated regulation of PDZK1 could be involved in this observation.

The idea of an impact of thyroid hormone status on PDZK1 and thereby urate homeostasis is further supported by findings, showing correlation of the polymorphisms rs1967017, rs1471633, and rs12129861 located in the PDZK1 promoter with the phenotype of uric acid levels in different populations (Yang et al., 2014,Kolz et al., 2009,Yang et al., 2010,Takada et al., 2014). Remarkably, we observed that the change from thymine to cytosine in position −4017 bp (rs1967017) resulted in a significantly reduced T3-mediated transactivation of the promoter. This polymorphism is located in the DR1 motif which we identified as most likely binding site for thyroid hormone receptors. We also showed a trend of lower PDZK1 mRNA expression in situ in renal tissues samples with this nucleotide exchange.

As mentioned before, regulation of the PDZK1 by THR may not only be of importance in the urate homeostasis, as the interaction of the scaffold protein with membrane transports is not limited to urate transporters. One of the protein shown to interact with PDZK1 is the sodium-hydrogen exchanger 3 (NHE3) (Sultan et al., 2013,Yeruva et al., 2015). This transporter is mainly involved in sodium reabsorption in kidney and intestine (Alexander and Grinstein, 2006), and it has been recently reported that a decrease in PDZK1 expression correlates with a reduced transport rate of NHE3 in enterocytes (Yeruva et al., 2015). Interestingly, T3 has also been shown to modulate NHE3 transport activity or renal expression of this transporter (Wang et al., 2005). This is in accordance with the fact that hyponatremia resulting from altered renal function is one of the symptoms linked to hypothyroidism (Montenegro et al., 1996,Nakahama et al., 2001). Based on our data, we hypothesize that THR-mediated regulation of PDZK1 may be a mechanistic explanation for this observation.

From a pharmacological point of view and regarding interindividual differences in drug response the enhancement of PDZK1 transcription by T3 is an important aspect, considering that PDZK1 interacts with, and modulates the function of drug transporters such as OATPs and ABC transporters (Walsh et al., 2015). Thus, an enhanced presence of their stabilizer PDZK1 would have an impact on the pharmacokinetics of their substrates. In detail, Oatp1a1, a member of the OATP family, has been reported to require PDZK1 for membrane surface expression in rat hepatocytes and thereby modulating transport rate (Wang et al., 2005). Similarly, knockdown of Pdzk1 in mice resulted in a decrease of Oatp1a1 abundance in the small intestine finally impairing the absorption of substrates (Sugiura et al., 2010). In this regard it is noteworthy that the last three C-terminal amino acids of the rodent Oatp1a1 are assumed to interact with PDZK1 (Sugiura et al., 2010). This peptide sequence is also present in the human OATP1A2 protein. Since rodent Oatp1a1 as well as OATP1A2 belong phylogenetically to the same subfamily of OATP transporters, it is not surprising that OATP1A2 has also been reported to interact with PDZK1 (Zheng et al., 2014). Focusing on intestinal drug transport, two representatives of the ABC transporter family have to be mentioned, namely ABCC2 (MRP2) and ABCG2 (BCRP) (Kocher et al., 1999,Shimizu et al., 2011,Walsh et al., 2015,Jedlitschky et al., 2006,Dietrich et al., 2003). However, studies on the influence of thyroid hormone status on transporter mediated drug absorption are limited (Burk et al., 2010,Shenfield, 1981).

Our experiments analyzing the impact of thyroid hormones on PDZK1 expression in Caco2 cells, which are a widely accepted as a cellular model of intestinal transport, not only showed induction of PDZK1 expression following treatment with T3 and T4. At this point it has to be mentioned that THRβ expression is assumed to be predominant in kidney and liver (Williams, 2000), while THRα is ubiquitously expressed (Flamant et al., 2006). However, we also show expression of THRβ in intestine and binding of THRβ to the PDZK1 promoter in Caco2 cells, indicating that THRβ-mediated regulation of PDZK1 also plays a role in enterocytes. Nonetheless, it cannot be excluded that the observed inducing effect of thyroid hormones on PDZK1 expression in Caco2 is attributed to activation of both THRβ and THRα, as it is known that both receptors are expressed in this cellular model. The previously reported interaction of PDZK1 with drug transporter and our finding suggests that modulation of PDZK1 expression by thyroid hormones T3 and T4 may be a mechanism influencing intestinal handling of drugs.

5. Conclusion

This study contributes to a better understanding of the effect of thyroid hormones in regulation of body function revealing PDZK1 as a target gene. The regulation of PDZK1 is of crucial interest in respect to its ability to interact with a variety of membrane proteins that are involved in different physiological processes including urate handling, electrolyte homeostasis, and pharmacokinetics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mce.2017.09.017.

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Disclosure statement

The authors have nothing to disclose.

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3.4 The scaffold protein PDZK1 modulates expression and function of the Organic Anion Transporting Polypeptide 2B1

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The scaffold protein PDZK1 modulates expression and function of the organic anion transporting polypeptide 2B1

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ABSTRACT

The protein family of Organic Anion Transporting Polypeptides (OATPs) summarizes various transporters known to facilitate cellular uptake of xenobiotics. One member of this family is OATP2B1. This transporter is ubiquitously expressed and possesses a PDZ-binding motif at the C-terminus. PDZK1 (PDZ domain-containing 1) is a scaffold protein that influences function of different membrane proteins by sorting/stabilization of their membrane localization. It was aim of the herein reported study to investigate whether there is an interaction between OATP2B1 and PDZK1, and to further characterize its impact on transport function. At first expression of both OATP2B1 and PDZK1 was evaluated in liver, kidney and intestine. Based on the existence of a C-terminal PDZ-class I binding motif in OATP2B1 and the co-expression in all tested tissues an interaction was likely. Testing the influence of PDZK1 on OATP2B1 transport function revealed enhanced transport capacity for estrone 3-sulfate, thereby suggesting a change in OATP2B1 amount in the membrane. This assumption was validated by Western blot analysis. Finally, deletion of the C-terminal PDZ-binding motif in OATP2B1 lowered the impact of PDZK1 on transport function. Taken together, we report an interaction of PDZK1 with OATP2B1, which influences localization and function of the transporter. Changes in PDZK1 expression may therefore be one factor contributing to interindividual differences in OATP2B1 mediated pharmacokinetic processes.

1. Introduction

Transfer through the plasma membrane is crucial for drugs to reach their site of action. It is well known that the process of crossing the membrane is facilitated by uptake and efflux transporters (Giacomini et al., 2010; Mandal et al., 2017). Accordingly, changes in membrane transporter expression and/or activity influence transmembrane transfer of substrate drugs and thereby pharmacokinetics, which finally impacts treatment outcome. One mechanism shown to modulate membrane protein expression and activity is the interaction with PDZ adapter proteins (Sugiura et al., 2011). Particularly, the protein family of Na^+/H^+ exchanger regulatory factors (NHERF), which summarizes various PDZ domain-containing scaffold proteins, has been reported to modify the function of membrane proteins (Walsh et al., 2015). The PDZ domains of NHERF proteins directly interact with specific amino acid sequences at the C-terminus of their target proteins thereby influencing their cellular sorting (Kalyoncu et al., 2010). The interplay with PDZ-containing proteins is assumed to not only stabilize the localization of target proteins (Shenolikar et al., 2002; Sugiura et al., 2011), but also to modulate their function due to the spatial arrangement of functional associated proteins in protein complexes (Kato et al., 2005; Sugiura et al., 2011; Weinman et al., 2007).

One member of the NHERF protein family is PDZK1 (NHERF3). This scaffold protein contains four PDZ domains, and has been shown to interact with the C-terminal residues of a variety of transporters involved in uric acid handling (Anzai et al., 2004; Kocher et al., 1998; Park et al., 2014). Accordingly, PDZK1 is assumed to be a determinant of the function of the so called “urate transportosome” (Anzai et al., 2012), which is a multi-protein complex composed of various uptake and efflux transporters localized in the apical membrane of the renal tubular cells (Anzai et al., 2012; van Aubel et al., 2002).

One transporter that is assumed to be part of this tubular transportosome, and that has been shown to be modulated by the direct interaction with PDZK1, is ABCC4 (MRP4) (Park et al., 2014). This member of the ATP-binding cassette (ABC) family exerts energy-dependent cellular efflux of uric acid, but also of nucleotide based drugs

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(Park et al., 2014; Van Aubel et al., 2005). Furthermore, ABCC4 is not only highly expressed in kidney, but also in other tissues of pharmacokinetic relevance including liver and intestine (Giacomini et al., 2010). Another ABC-transporter exhibiting a C-terminal class 1 PDZ-binding motif that has been shown to interact with the fourth PDZ domain of PDZK1 is ABCC2 (Emi et al., 2011; Kocher et al., 1999). Considering that ABCC2 is a major determinant in renal, hepatic and intestinal handling of products of the phase II biotransformation (Jedlitschky et al., 1997; Suzuki and Sugiyama, 2002) further provides evidence that PDZK1, even if highly expressed in kidney, executes its organizational role also in other tissues with polarized cells. This assumption is further supported by the presence of PDZK1 in liver and intestine (Kocher et al., 1998).

Intestinal handling of drugs is modulated by uptake and efflux transporters. In terms of cellular uptake, it is assumed that members of the solute carrier (SLC) superfamily are of relevance. One subfamily of SLC transporters is the family of Organic Anion Transporting Polypeptides (OATPs) (Kalliokoski and Niemi, 2009; Shitara et al., 2013). The first OATP, shown to be modulated by PDZK1 is OATP1A2 (Zheng et al., 2014). Even if OATP1A2 was reported to be present in intestine, its function is assumed to be of highest relevance in the blood-brain-barrier (Glaeser et al., 2007; Meier et al., 2007; Steckelbroeck et al., 2004). Another OATP, reported to be functional in liver and intestine is the ubiquitously expressed OATP2B1 (Kobayashi et al., 2003; Kullak-Ublick et al., 2001). This transporter facilitates sodium-independent uptake of endogenous sulfated anionic conjugates as well as of drugs such as HMG-CoA reductase inhibitors (Grube et al., 2006b; Kullak-Ublick et al., 2001; Tamai et al., 2001).

Most of the clinical studies investigating the intestinal function of OATP2B1 focused on its luminal localization whereby referring to findings showing OATP2B1 in the apical membrane of enterocytes and Caco-2 cells (Kobayashi et al., 2003; Sai et al., 2006). However, a recent report by Keiser et al. states conflicting data suggesting basolateral enrichment of OATP2B1 in jejunum and Caco-2 cells (Keiser et al., 2017). Nonetheless, among the OATPs present in enterocytes, OATP2B1 is assumed to be dominant in drug handling (Tamai, 2012). Consequently, understanding factors modulating transport activity of OATP2B1 would contribute to an improved comprehension of oral drug absorption. One of these modulating factors could be the interaction with PDZ domain-containing proteins, like PDZK1. Considering that OATP2B1 exhibits a carboxyl-terminal class I PDZ-binding motif it was aim of the herein reported study to investigate whether there is an interplay between OATP2B1 and PDZK1.

2. Material and methods

2.1. Materials

Unless stated otherwise chemicals and media were purchased from Sigma Aldrich, Buchs, Switzerland. Bovine serum albumin, Citric acid, DAPI (4′,6-diamidino-2-phenylindole), EDTA, glycerol, glycin, hematoxylin (hemalum solution acid acc. to Mayer), HEPES, H_2O_2, KCl, KH_2PO_4, 2-mercaptoethanol, NaCl, Na_2HPO_4, sodium citrate, SDS, Tris-HCl, Tween 20, Roti-Mount Fluor Care and Rotiszint®eco Plus were obtained from Carl Roth, Arlesheim, Switzerland.

2.2. Cell culture

Madin-Darby canine kidney (MDCKII, ATCC® CRL-2936™) and cervix carcinoma HeLa (ATCC® CCL-2™) cells originally obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM, D6429) supplemented with 10% fetal calf serum (FCS, AMIMED, BioConcept Ltd., Allschwil, Switzerland) and 1% GlutaMAX™ (Thermo Fisher Scientific, Reinach, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO_2. MDCKII-OATP2B1 cells (Grube et al., 2006b) were cultured in presence of Hygromycin B (Roche, Basel, Switzerland) at a final concentration of 0.4 mg/ml to maintain continuous selection.

2.3. Human tissue samples

Human kidney tissue samples from patients undergoing surgery of renal carcinoma were obtained after written informed consent and approval by the local ethics committee of the Medical Faculty of the University of Greifswald (III UV 12/03). Isolation of RNA and protein of the kidney samples was previously described (Prestin et al., 2017b). The paraffin embedded tissue blocks from kidney, intestine and liver were purchased from AMS Biotechnology (Bioggio-Lugano, Switzerland). Tissue sections (7 µm) were prepared using the microtome HM 340E (Thermo Fisher Scientific, Zug, Switzerland) and mounted on glass slides, dried over night at 37 °C, and stored at 4 °C until further use.

2.4. Quantitative polymerase chain reaction (qPCR)

The total RNA of kidney, small intestine, and liver was purchased from AMS Biotechnology and 1000 ng of each RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (LuBioScience). The resulting cDNA (10 ng/µL) was applied to real-time PCR using the commercially available TaqMan® Gene Expression assays (Thermo Fisher Scientific, Reinach, Switzerland) for detection of OATP2B1 (*SLCO2B1,* Hs01030343_m1), PDZK1 (*NHERF3*, Hs00275727_m1) and 18SrRNA (H s4319413E), and the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Here, ΔCt was the difference of the Ct-values (threshold of cycle) of the target gene and the gene used for normalization (18S ribosomal RNA). The ΔCt value of each sample was related to the mean ΔCt-value of the indicated control, resulting in the ΔΔCt-value, which was then potentiated to the base of two ($2^{-\Delta\Delta Ct}$). No template controls were included in all analyses.

2.5. Immunohistochemistry

Staining started with the removal of paraffin in two changes of xylene followed by rehydration in a declining ethanol series. Heat induced epitope retrieval was performed autoclaving the tissue for 20 min in citrate buffer (pH 6.0) or in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA; pH 9.0). After heating, the samples were transferred to ice-cold distilled water, washed with phosphate-buffered saline (PBS), and the activity of endogeneous peroxidase was quenched by incubation in 3% H_2O_2 in methanol for 20 min. After blocking in 5% FCS - PBS the slides were incubated with the primary antibody at 4C° overnight. The anti-PDZK1 (ab92491; abcam), and the anti-OATP2B1 antibody (Grube et al., 2006a) were used at a dilution of 1:50 in blocking solution. Before and after the incubation with the secondary antibody (diluted 1:200) for 2 h at room temperature, the tissue sections were washed several times with PBS. The slides were then pre-incubated for 10 min with 50 mM Tris-buffer (TB, pH 7.6) at 37 °C before being treated with freshly prepared DAB solution containing 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Thermo Fisher Scientific) in TB supplemented with 0.02% H_2O_2. The nuclei were stained with Mayer's hematoxylin and mounted using Aquatex (Merck & Cie, Schaffhausen, Switzerland). Staining was imaged under bright-field illumination using the Leica DMi8 microscope (Leica, Heerbrugg, Switzerland) equipped with the MC10 HD camera (Leica). Images were acquired and processed with the LAS (Leica Application Suite) software version 4.8 (Leica).

2.6. Immunofluorescence microscopy

The paraffin embedded tissue sections were deparaffinized and rehydratized as described above. After heat induced epitope retrieval and

three washing steps with water, the slides were blocked with 10% FCS and 0.4% Tween in PBS- for one hour, followed by incubation with anti-OATP2B1 ((Grube et al., 2006a), diluted 1:100) or anti-PDZK1 antibody (ab125094, abcam, diluted 1:25) over night at 4 °C. Then the slides were washed 3 times with PBS followed by incubation for 1 h with the respective fluorescent-labelled antibodies Alexa Fluor 488-labelled immunoglobulin G (1:200; Life Technologies) and Alexa Fluor 568-labelled immunoglobulin G (1:100; Life Technologies). Slides were washed and mounted with Roti-Mount Fluor Care containing DAPI for counterstaining of the nuclei. Staining was imaged and processed with a LSM 780 system and the ZEN software (Zeiss, Jena, Germany).

2.7. GEO (gene expression omnibus) data sets

mRNA expression data for analysis of the correlation between PDZK1 and OATP2B1 were selected from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Search criteria were the relevant tissue, mRNA expression profiling, and *Homo sapiens*, whereby resulting in 216 entries for kidney, 734 entries for liver and 85 entries for small intestine. From those, data sets reporting on at least 5 tissue samples from healthy individuals were selected for further statistical analysis using GraphPad Prism (version 6, GraphPad Software Inc., La Jolla, CA, USA).

2.8. Generation of the adenovirus encoding for PDZK1

The coding sequence of PDZK1 [NM002614.4] was amplified using primers (PDZK1_for 5′-CCTCCAGAAATGACCTCCACCTTCAAC-′3 and PDZK1_rev 5′-TTGTTTTCATCACATCTCTGTATCTTCAG-′3) and the AmpliTaqGold™ kit (Thermo Fisher Scientific). The PCR-amplicon was purified and inserted in pEF6-V5/His TOPO (Invitrogen, Themo Fisher Scientific). After plasmid amplification and sequence control the coding sequence was transferred into pENTR-1A (Invitrogen, Themo Fisher Scientific), which was basis for generation of the respective adenovirus as described by the manufacturer (ViraPower™ Adenoviral Gateway™ Expression Kit, Invitrogen, Thermo Fisher Scientific). In detail, the PDZK1-pENTR1A was used to transfer the coding sequence into pAd/CMV/V5-DEST™ Gateway Vector by recombination. Then the plasmid (pAd-PDZK1) was transfected in HEK293A cells (Thermo Fisher Scientific) for generation and purification of the adenovirus Ad-PDZK1. Ad-lacZ was generated using the commercially obtained plasmid (pAd/CMV/V5-GW/lacZ, Thermo Fisher Scientific). Generation and characterization of Ad-OATP2B1 and Ad-GFP have previously been reported (Knauer et al., 2010; Meyer Zu Schwabedissen et al., 2014). Finally, plaque forming units (pfu) in the cell lysate were determined using the Adeno-X™ Rapid Titer Kit (Takara Clontech, Saint-Germain-en-Laye, France).

2.9. Enrichment of the membrane fraction of cultures cells

For comparison of protein abundance in the intracellular and membrane fraction after adenoviral infection, MDCKII and MDCKII-OATP2B1 were seeded in 100 mm dishes at a density of 1×10^6 cells per plate and infected with 50 pfu/cell Ad-PDZK1 or Ad-EGFP. Twentyfour hours post-infection the medium was changed. The day after, cells were washed with PBS and lysed in 5 mM Tris-HCl (pH 7.4) supplemented with protease inhibitor cocktail (PIC). After five cycles of freezing and thawing the membrane fraction was enriched by ultracentrifugation at 100,000 ×*g* and 4 °C for 30 min. The supernatant was collected as intracellular fraction and the pellet was resuspended in 5 mM Tris-HCl-PIC. Subsequently, protein content was determined using the Bicinchoninic acid (BCA) protein assay (Pierce™, Thermo Fisher Scientific) and the Infinite M200PRO plate reader (Tecan, Männedorf, Switzerland).

2.10. Western blot analysis

Protein expression was detected by immunoblot analysis using 10 µg protein of each sample. Samples were separated by 7.5% SDS-PAGE and proteins were electro-transferred onto a nitrocellulose membrane using a tank blotting system (Mini Trans-Blot® Cell; Bio-Rad Laboratories AG, Cressier FR, Switzerland). After blocking with 5% FCS-Tris buffered saline supplemented with Tween (TBS-T; 25 mM Tris, 140 mM NaCl, 2.6 mM KCl, 0.4% Tween 20) the membranes were incubated with the primary antibody overnight at 4 °C. The primary antibodies used for detection of OATP2B1 and PDZK1 were anti-OATP2B1 (Grube et al., 2007) and anti-PDZK1 (ab92491, abcam, Cambridge, UK) at a dilution of 1:5000 and 1:2500 in TBS-T with 5% BSA, respectively. As loading controls for the cellular fractions the antibodies anti-calnexin (ADI-SPA-865, Enzo life Science Lausen, Switzerland), or anti-actin (sc-1616, Labforce AG, Muttenz, Switzerland) were used at a dilution of 1:1000 in TBS-T with 5% BSA. After several washing steps with TBS-T, membranes were probed for 1 h at room temperature with the respective secondary horseradish peroxidase (HRP) conjugated antibodies (BioRad Laboratories, Cressier, Switzerland). Enzymatic activity was visualized using the Western ECL Plus Substrate (Thermo Fisher Scientific) and detected using the ChemiDoc™ MP System (BioRad Laboratories). Densitometry was performed with the Image Lab software (Version 4.1; BioRad Laboratories).

2.11. Transport experiments (adenoviral)

To study the influence of PDZK1 on OATP2B1 function, MDCKII and MDCKII-OATP2B1 cells were seeded in 24-well plates. The day after seeding the cells were transduced with 50 pfu/cell of Ad-PDZK1 or Ad-lacZ. After 24 h the medium was changed and the cells were kept in culture for an additional day. To determine the cellular accumulation the cells were incubated for 10 min at 37 °C with increasing concentrations of estrone 3-sulfate (E_1S) in incubation buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.5 mM $CaCl_2$ *H_2O, 1.2 mM $MgSO_4 * 7H_2O$, 5 mM glucose and 12.5 mM HEPES, pH 7.4) supplemented with [^{3}H]-E_1S (100, 000 dpm per well; Hartmann Analytic GmbH, Braunschweig, Germany). After incubation, cells were washed three times with ice-cold PBS, lysed in 0.2% SDS-5 mM EDTA and the protein content was determined by BCA assay. The remaining cell lysate was mixed with 2 ml scintillation fluid (Rotiszint® eco plus) and radioactivity was quantified using a liquid scintillation β-counter (Tricarb 2900 TR; TOPLAB, Rickenbach, Switzerland).

2.12. Generation of OATP2B1 expression vector missing the PDZ-binding motif

The deletion variant of OATP2B1, which lack the three last C-terminal amino acids (Del) was generated by mutation of the amino acid S707 to ×707 using the vector pEF6-OATP2B1 (kindly provided by Prof. Richard Kim (Tirona et al., 2003)), the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies Schweiz AG, Basel, Switzerland), and the primer 5′-GGGAAGAAGCCAGAGGATTAACGAG TGTGAAGGGCA-3′ (MicroSynth AG, Balgach, Switzerland) according to the manufacturers protocol.

2.13. Transport experiment missing the PDZ-binding motif

Hela cells were seeded at a density of 75,000 cells/well in 24 well plates. Twentyfour hours after seeding, cells were transfected (250 ng/plasmid/well) with pEF6-PDZK1 in presence of pEF6-OATP2B1 wild-type (WT) or pEF6-OATP2B1 (Del) using jetPRIME® transfection reagent (Polyplus, Chemie Brunschwig, Basel, Switzerland) according to the manufacturer's instructions and the recombinant vaccinia virus (vTF-7, (Kim et al., 1999)). Briefly, cells were once washed with pre-warmed PBS and DMEM without supplements before incubating 30 min

at 37 °C with vTF-7 (7 plaque-forming unit/cell). Cells were washed with DMEM without supplements followed by addition of complete growth medium. The mixture of vectors and jetPRIME® (using a ratio of 500 µg vector to 1 µl reagent) was added to the media and incubated overnight. The day after the cells were washed with DMEM and 24 h later the cells were washed with pre-warmed PBS before being exposed to an increasing E_1S concentration (up to 50 µM) supplemented with [^{3}H]-E_1S (100,000 dpm/well) for 10 min. Sample lysis and quantification was performed as written in the section on transport experiments.

2.14. Foerster resonance energy transfer (FRET)

Wild-type and OATP2B1-ECFP-expressing MDCKII cells were seeded on Nunc™ Lab-Tek™ chambered coverglass slides (Nunc™ Lab-Tek™ Thermo Fisher Scientific, Waltham, USA City Country). PDZKI was subcloned from pEF6-PDZK1 vector into the pEYFP expression vector (Takara ClontechBio USA, City CountryMountain View, USA). Two different EYFP-labelled variants of PDZK1 were generated. One with EYFP linked to the N-terminus of PDZK1 (EYFP-PDZK1) and one where the sequence encoding for EYFP was attached to the C-terminus of the scaffold protein (PDZK1-EYFP). Cells stably transfected with OATP2B1-ECFP were transfected with one of these plasmids or the pEYFP vector control using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, USA). After 24 h transfection FRET was assessed using a Zeiss LSM 780 confocal microscopesystem (Carl Zeiss Microscopy GmbH, Jena, Germany). In detail, FRET analyses were carried out using with a 100× oil immersion objective and a LASOS LGN 3001/Zeiss AIM-SYSTEM 2504000156 argon ion laser unit (LASOS Lasertechnik GmbH, Jena, Germany). In order to acquire FRET via sensitized emission, the cells were imaged through three filter sets including the ECFP filter set (exc. 458 nm; em. 459–514 nm), the EYFP filter set (exc. 514 nm; em. 515–646 nm) and the FRET filter set (exc. 458 nm; em. 515–646 nm). Acquisition and data analysis were performed with the Zeiss ZEN 2010 software (Carl Zeiss Microscopy GmbH, Jena, Germany). FRET was calculated using the N FRET method [39]. For evaluation only the cell membrane was considered. At least five cells with a varying number of membrane regions were taken into calculation for each transfected expression vector.

2.15. Statistical analysis

Data are presented as mean ± SD. Data analysis was performed using the GraphPad Prism software (version 6, GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). A p-value below 0.05 was considered as statistically significant. The applied statistical test is mentioned in the context of the results. The Michaelis-Menten constant (K_m) and maximum transport velocity (V_{max}) were estimated using GraphPad applying a nonlinear regression of the equation: $Y = \frac{(V_{max} \times x)}{(K_m + x)}$, where Y is the transport velocity per time and x is the E_1S substrate concentration.

3. Results

3.1. Expression of OATP2B1 and PDZK1 in different human tissues

At first we verified the co-expression of OATP2B1 and PDZK1 in various human tissues. Comparable amounts of OATP2B1 mRNA (Fig. 1A) were detected in intestine, liver and kidney, while PDZK1 mRNA expression was about 10-fold higher in kidney compared to liver and intestine (Fig. 1B). However, the herein assumed interaction of PDZK1 and OATP2B1 would imply a co-localization of the proteins. Accordingly, localization of OATP2B1 and PDZK1 was determined in the abovementioned tissues by immunohistochemistry. Both proteins were shown to be present in the investigated tissues. In detail, OATP2B1 and PDZK1 were located at the luminal site of enterocytes and in the sinusoidal membrane of hepatocytes (Fig. 2A). In kidney, OATP2B1 was either located in the basolateral or in the luminal membrane of renal tubular cells. Importantly, both PDZK1 (Fig. 1C) and OATP2B1 (Fig. 1 D) were shown to be located in the luminal membrane of proximal tubular cells (OATP2B1, PDZK1, Fig. 1H), suggesting that PDZK1 influences polar sorting of OATP2B1 in this tissue. Furthermore, we tested whether there is a correlation of both genes in these tissues. Basis for this analysis were publically available mRNA expression data accessible at the GEO-website. Analyzing data-sets summarizing at least n = 5 samples indicated by the authors as healthy tissues revealed a direct correlation for both genes in small intestine (Fig. 2B, GEO Series GSE13083, R2 = 0.851, p-value = 0.0257; n = 5, (Stairs et al., 2008)), liver (Fig. 2C, GEO DataSets GDS3883, R2 = 0.93, p-value = 0.0005; n = 7, (Misu et al., 2010)), and kidney (Fig. 1E, GSE83095, R2 = 0.8272, p-value < 0.0001; n = 16). The correlation was further supported analyzing mRNA (Fig. 1F) and protein (Fig. 1G) expression data obtained in healthy kidney samples performing a Pearson correlation.

3.2. Impact of PDZK1 on OATP2B1 activity and expression in MDCKII cells

The correlation of mRNA expression of both genes in human kidney and the detection of PDZK1 and OATP2B1 at the luminal membrane of the tubular cells supported the idea of an interaction between the scaffold protein and the transporter. In order to test, whether expression of PDZK1 influences OATP2B1 activity we conducted transport experiments using MDCKII cells stably expressing human OATP2B1 (MDCKII-OATP2B1). For expression of PDZK1 those cells were infected with Ad-PDZK1, an adenovirus encoding for the scaffold protein. Ad-lacZ served as control. Testing the cellular uptake of E_1S a known substrate of OATP2B1 revealed a significantly enhanced V_{max} in presence of PDZK1 (V_{max} mean ± SE, 138.0 ± 12.32 fmol ∗ µg protein^{-1} ∗ min^{-1}) in comparison to the control cells (108.7 ± 11.2 fmol ∗ µg protein^{-1} ∗ min^{-1}; Student's *t*-test p-value = 0.009; data not shown). Importantly, no change in affinity was observed calculating the respective K_m-values (mean ± SE, Ad-lacZ vs. Ad-PDZK1, 6.36 ± 2.35 µM vs. 6.81 ± 2.14 µM, Student's t-test p-value = 0.61), thereby suggesting a change in abundance of functional transporter at the membrane. Western blot analysis revealed that presence of PDZK1 significantly augmented the amount of transporter in the membrane fraction (Fig. 3A). Densitometric analysis showed an about 1.7-fold higher abundance of OATP2B1 in the membrane fraction after co-expression of PDZK1 (Fig. 3B). In addition, we were able to also detect PDZK1 in the enriched membrane fraction of MDCKII-OATP2B1, this was not the case in Ad-PDZK1 transfected MDCKII cells (data not shown).

3.3. Physical proximity of OATP2B1 and PDZK1

Hitherto, our data clearly suggest an impact of PDZK1 on localization and function of OATP2B1; however, this finding could still be of indirect nature. To verify an interaction between scaffold protein and transporter we assessed the fluorescence resonance energy transfer (FRET) of labelled OATP2B1 and PDZK1 in MDCKII cells. In detail, MDCKII cells overexpressing human OATP2B1 C-terminally tagged with enhanced cyan fluorescence protein (ECFP) were transiently transfected with PDZK1 which was N-terminally or C-terminally tagged with enhanced yellow fluorescent protein (EYFP-PDZK1 or PDZK1-EYFP, respectively. The calculated FRET efficiency was significantly higher in presence of PDZK1-EYFP (mean FRET efficacy efficiency ± SD, 0.147 ± 0.036, Dunnett's multi-comparisons p-value < 0.0001) compared to N-terminally tagged EYFP-PDZK1 (0.086 ± 0.028, p-value = 0.906), or EYFP-control (0.087 ± 0.027) (Fig. 4A & B).

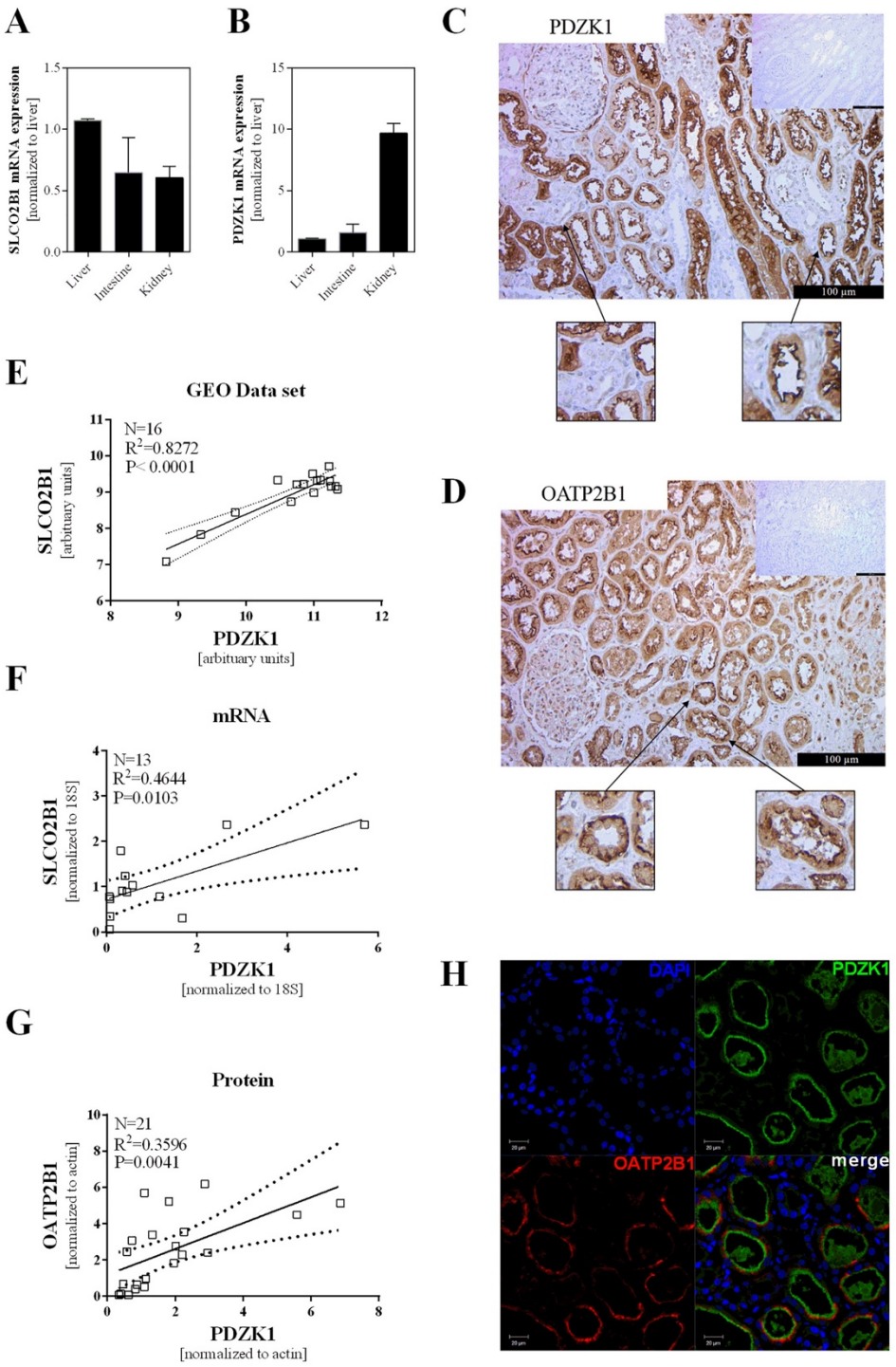

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Fig. 1. Detection of OATP2B1 and PDZK1 expression in different human tissues. The amount of SLCO2B1 (A) or PDZK1 mRNA (B) in human liver, intestine and kidney was determined by real time-PCR. Data are presented as mean ± SD (geb = 3 in duplicates). Localization of PDZK1 (C) and OATP2B1 (D) in kidney was detected by immunohistochemical staining. (E–G) Correlation of PDZK1 and OATP2B1 mRNA expression in a selected GEO data set and healthy kidney samples were statistically analyzed performing Pearson correlation. (F) Correlation of PDZK1 and OATP2B1 protein expression in healthy kidney samples assessed by Western blot analysis. Data were analyzed performing Pearson correlation. (H) Localization of PDZK1 (green) and OATP2B1 (red) in kidney was detected by immunofluorescent staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

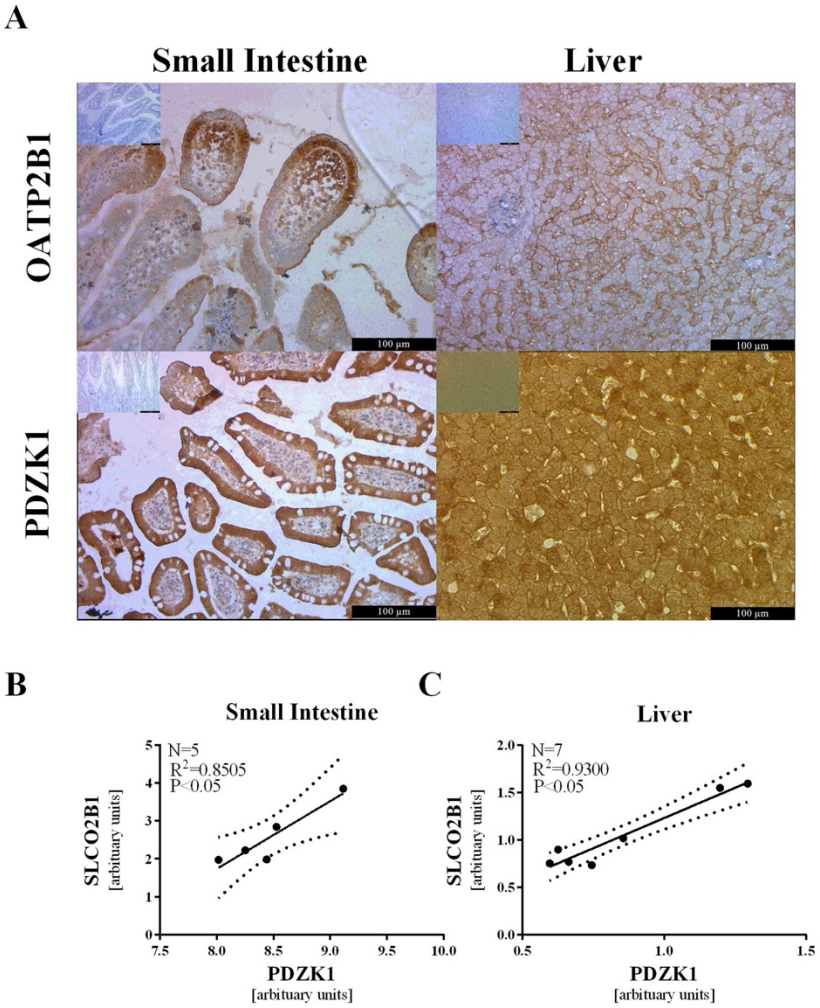

Fig. 2. Immunohistochemically staining and co-expression of PDZK1 and OATP2B1 in small intestine and liver. (A) Localization of PDZK1 and OATP2B1 was analyzed with the antibodies anti-PDZK1 or anti-OATP2B1. Noteworthy, heat induced epitope retrieval was performed in Citric-buffer (pH 6) in all tissues except for the detection of PDZK1 in liver where we used TE-Buffer (pH 9). (B, C) Basis for the correlation analysis were expression data published in the GEO-database at NCBI. GEO data sets (B: GSE 13083 intestine; C: GDS3883 liver) were statistically analyzed performing Pearson correlation.

3.4. Deletion of the OATP2B1 C-terminus affects the maximal transport velocity of E_1S

Based on the current knowledge, PDZK1 is assumed to directly bind to C-terminally located amino acids of membrane proteins summarized as the so called PDZ-binding motif, which in general consists of S/T-X-L/V-COOH (Kato et al., 2004), where X can be any amino acid. In human OATP2B1 this C-terminal PDZ-binding motif would be the amino acid sequence Serine-Arginine-Valine illustrated as red triangle in Fig. 5A. In order to further support the notion that PDZK1 directly binds to OATP2B1 we generated an OATP2B1 deletion variant lacking the C-terminal amino acids and tested the influence of PDZK1 on transport activity. Here, Hela cells were transiently transfected with a plasmid encoding for wild-type OATP2B1 (WT), or for the PDZ-binding

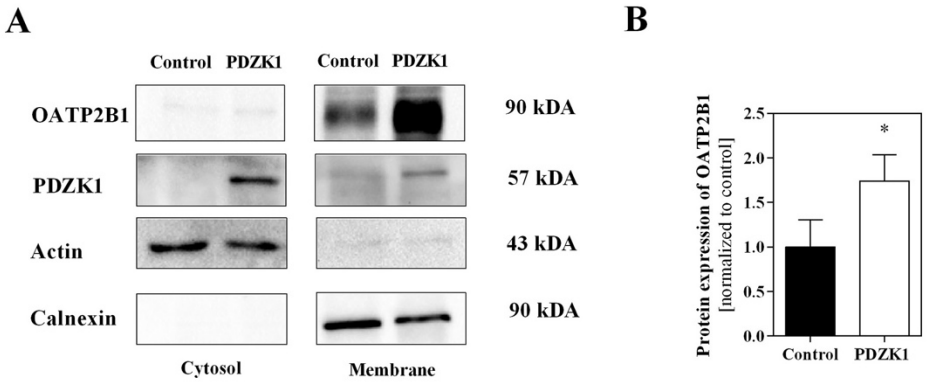

Fig. 3. Impact of PDZK1 on OATP2B1 on protein level. (A) Protein expression in the cytosolic or the membrane fractions enriched after adenoviral transfer of PDZK1 or control to MDCKII-OATP2B1 cells was determined by Western blot analysis. (B) Amount of protein in the membrane fraction was densitometrically analyzed, normalizing the amount of OATP2B1 to that of calnexin. Data are presented as mean ± SD, n = 3 independent experiments. *p-value < 0.05 unpaired *t*-test.

motif deletion variant of the transporter (Del). The increase in V_{max} in presence of the scaffold protein previously shown in MDCK-OATP2B1 was also observed in Hela cells transfected with WT and PDZK1 (mean V_{max} ± SD; PDZK1: 443.8 ± 128.2 fmol * µg protein^{-1} * min^{-1}; control: 233.9 ± 50.09fmol * µg protein^{-1} * min^{-1}; p-value = 0.0371; Fig. 5B). The comparison of WT or Del co-transfected with PDZK1 revealed a statistically significant reduction in V_{max} (mean V_{max} ± SD; OATP2B1 Del: 223.2 ± 53.24 fmol * µg protein^{-1} * min^{-1}; p-value = 0.0039; Fig. 5C), while the K_m-values did not differ significantly even though a trend was observed (mean ± SD, WT vs. Del, 162.7 ± 95.45 µM vs. 37.85 ± 12.35 µM, Tukey's multiple comparisons test p-value = 0.0619).

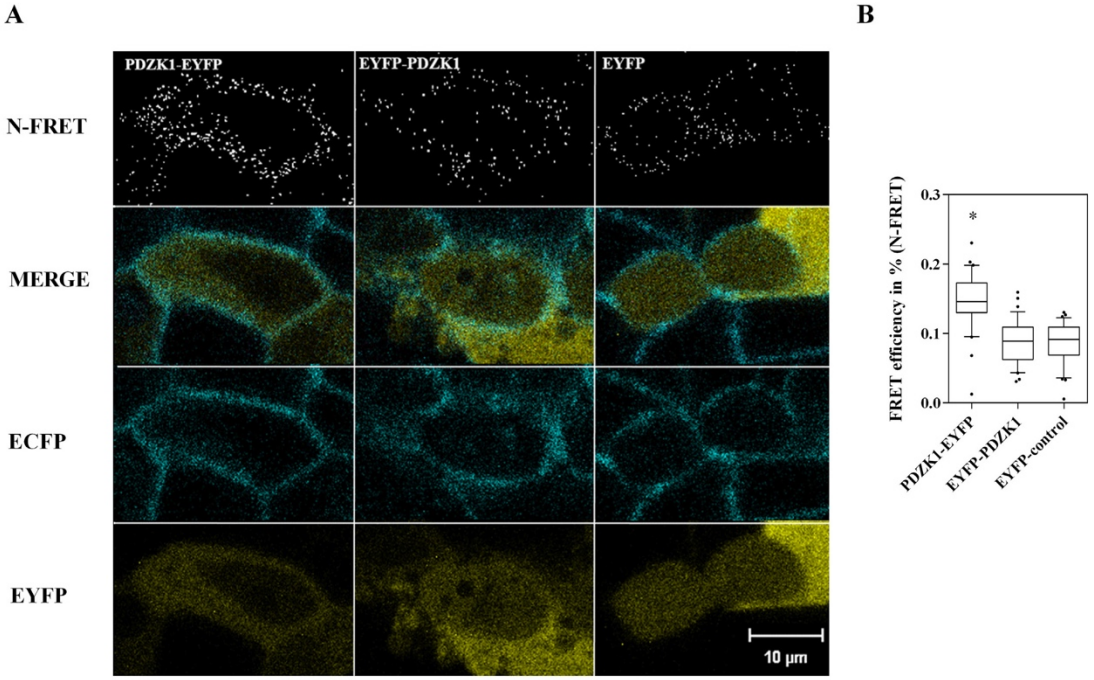

Fig. 4. FRET analysis of the direct interaction between PDZK1 and OATP2B1. (A) Images of MDCK-OATP2B1-ECFP cells transiently transfected with the PDZK1-EYFP, the EYFP-PDZK1, or pEYFP vector control. The shown images were captured either through the N-FRET, the ECFP, or/and the EYFP filter set. (B) The horizontal line in the box presented the median, the box boundaries represented 25 and 75 percentiles while the whiskers showed the 5 to 95 percentile. The FRET efficiency was calculated with the N-FRET method (n = 3 independent experiments in technical replicates ≥20). For statistical analysis the Dunnett's multi comparisons test was used * p-value < 0.05.).

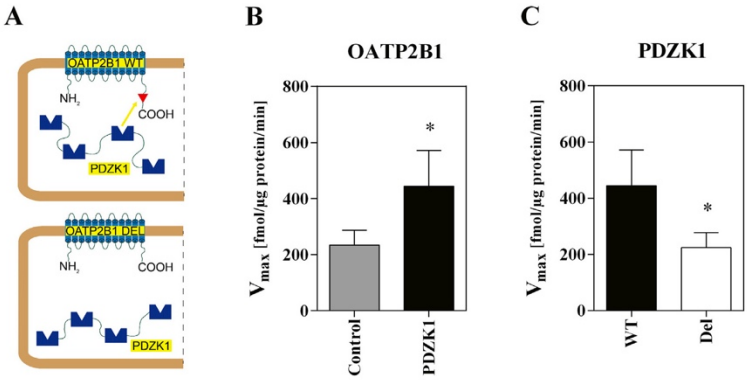

Fig. 5. Assessment the transport activity after deletion of the PDZ-binding motif. (A) Schematic illustrating the interaction of the C-terminal PDZ-binding motif (red triangle) of OATP2B1 (OATP2B1, upper panel) with PDZK1, and without the binding motif (OATP2B1-DEL; lower panel). (B) Comparison of the maximal transport rate (V_{max}) in presence or absence of PDZK1. Data were obtained after transient expression in Hela cells. (C) The effect of the C-terminal binding motif was determined assessing the maximal transport velocity for E_1S in Hela cells transfected with PDZK1 and either wild-type OATP2B1 (WT) or its deletion variant (DEL). Data are presented as mean ± SD (n = 3 in technical triplicates). For statistical analysis the Tukey's multiple comparisons test was used * p-value < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Taken together, our data suggest that localization and function of OATP2B1 are modulated by direct interaction with PDZK1 (NHERF3). As mentioned before, the latter is a scaffold protein, which has been shown to directly bind to a variety of membrane proteins including receptors and transport proteins (Hu et al., 2013; Park et al., 2014; Walther et al., 2015; Zheng et al., 2014), whereby stabilizing membrane proteins, and affecting their function (Claperon et al., 2011; Kato et al., 2006; Sugiura et al., 2006b). Investigating the effect of PDZK1 on OATP2B1 transport function revealed an increase in maximal transport velocity (V_{max}), but no change in the Michaelis-Menten constant (K_m) for E_1S. The augmentation in V_{max} without impact on binding affinity suggested a change in protein amount in the membrane. This assumption was supported by our finding showing an about 50% increase of OATP2B1 in the plasma membrane in presence of PDZK1 in MDCKII cells. Our observation is in accordance with findings on the interaction of PDZK1 with OAT4 (SLC22A11) (Miyazaki et al., 2005), URAT1 (SLC22A12) (Anzai et al., 2004), or OATP1A2 (SLCO1A2) (Zheng et al., 2014), also showing an increase in V_{max} without change in affinity. In 2011 Sugiura et al. (Sugiura et al., 2011) categorized PDZ domain-containing proteins by their impact on membrane proteins: as modulators of the sorting, as stabilizers of the proteins on the membrane, as regulators of transport activity, or as facilitators of signal transduction. In their opinion PDZK1 contributes to all categories as it has been shown to influence the sorting and stabilization of ABCC2 (MRP2) (Emi et al., 2011), the activity of OCTN2 (Kato et al., 2005), and as it facilitates the phosphorylation of CFTR by cAMP orchestrating a protein complex with ABCC4 (MRP4), which is a known efflux pump for cAMP (Li et al., 2007). Furthermore, PDZK1 has been reported to exert a stabilizing and/or a sorting effect on OAT4, URAT1 and OATP1A2 (Anzai et al., 2004; Miyazaki et al., 2005; Zheng et al., 2014) as mentioned above. We suggest a similar impact of PDZK1 on OATP2B1 reasoned by the increased amount of OATP2B1 in the plasma membrane in presence of the scaffold protein.

The interaction of membrane proteins with PDZK1 depends on the presence of a PDZ-binding motif, which has to be located at the intracellular C-terminus of the targeted protein (Malmberg et al., 2008; Park et al., 2014). In an early screening of various C-terminal sequences of OATPs for interaction with PDZK1 Kato et al. reported no interaction for PDZK1 with OATP2B1 (Kato et al., 2004). However, even if the authors observed interaction with the C-terminus of other OATPs, now validated as interacting partners of PDZK1 (Kato et al., 2005; Zheng et al., 2014; Zhou et al., 2008), it remains to be noted that the authors used a yeast two hybrid system applying synthetic peptide sequences and yeast cells (AH109 strain), which might have influenced the interaction. In order to verify direct interaction of OATP2B1 with PDZK1, we tested whether deletion of the class 1 PDZ-binding motif at the C-terminus influences the observed effect on the maximal transport rate for E_1S. In accordance with findings by Zheng et al., we observed that the deletion of the C-terminal PDZ-binding motif of OATP2B1 significantly diminished the alteration in E_1S V_{max} by the scaffold protein (Zheng et al., 2014).

Direct interaction of PDZK1 with OATP2B1 is also supported by the findings in the herein performed FRET analysis using EYFP-labelled PDZK1, and ECFP-labelled OATP2B1. In our experiments we observed energy transfer in presence of the C-terminally labelled PDZK1-EYFP, but not in presence of the N-terminally labelled EYFP-PDZK1. FRET analyses have been applied before to investigate the interplay of NHERF family members and proteins with a PDZ-binding motif (Giral et al., 2011; Kunkel et al., 2009). For the sodium-dependent phosphate transporters 2a (NaPi-2a) and 2c (NaPi-2c), the authors observed higher FRET efficiency for NaPi-2c, than for NaPi-2a, and explained their finding by the distance of EYFP-PDZK1 to the ECFP-labelled N-terminus of the respective transporter. Importantly, it had previously been demonstrated that NaPi-2c interacts with the second PDZ domain of PDZK1 (Villa-Bellosta et al., 2008), while NaPi-2a binds to the third PDZ domain, whereby resulting in a modification of the distance of the fluorophores (Hernando et al., 2005). Considering these findings, we hypothesize an involvement of a PDZ-domain near the C-terminus of PDZK1 in the interaction with the C-terminus of OATP2B1.

However, there might be tissue specificity in the modulation of target protein by scaffold proteins, as previously reported for OAT4. In detail, in renal cell lines from pigs (LLC-PK1) and monkeys (COS-7) stably transfected with human OAT4, membrane stabilization of the transporter by PDZK1 was observed, while no such effect was detected in BeWo cells originating from human placenta (Zhou et al., 2008). One might hypothesize that the lack of modulation is due the fact that BeWo cells are not polarizing in culture. In this regard it is of importance to note that most in vitro studies on the role of scaffold proteins have been performed in non-polarized cells such as HEK293, or HeLa cells (Anzai et al., 2004; Kato et al., 2005; Miyazaki et al., 2005; Park et al., 2014; Sugiura et al., 2006a; Zheng et al., 2014). In our study we tested both polarized (MDCKII) and non-polarized cells (HeLa), and observed the influence of PDZK1 on OATP2B1 in both cell lines.

Furthermore, we showed expression of both mRNA and protein of OATP2B1 and PDZK1 in organs crucial for drug absorption and elimination namely intestine, liver and kidney. Importantly, we not only observed co-expression and correlation of the amount of both gene products, but also a localization of both proteins at the same pole of polarized cells in these tissues. In liver, our observation is in accordance with previous findings by others showing localization of OATP2B1 and

PDZK1 in the sinusoidal membrane of the hepatocytes (Ikemoto et al., 2000; Kopplow et al., 2005). In intestine we observed expression of both proteins at the luminal pole of enterocytes. However, for OATP2B1 there is a report suggesting basolateral expression, which clearly contradicts findings by others (Keiser et al., 2017; Kobayashi et al., 2003). For intestinal PDZK1 there is only information in the literature on apical localization in enterocytes of mice (Malmberg et al., 2008; Sugiura et al., 2010). Interestingly, in kidney we observed OATP2B1 in the basolateral membrane of epithelial cells of the distal tubuli and in the apical membrane of proximal tubular cells. Importantly, PDZK1 is only present in the apical membrane of proximal tubuli (Biber et al., 2005; Giral et al., 2011; Gisler et al., 2001; Kocher et al., 1998; Prestin et al., 2017a; Pribanic et al., 2003; Thomson et al., 2005), thereby suggesting that PDZK1 controls subcellular localization in this organ. In terms of transport proteins, the influence of PDZK1 on transport function has particularly been shown in kidney. In this context Park et al. reported that ablation of Pdzk1 in mice significantly decreased the amount of Mrp4 in the apical membrane of the proximal tubular cells (Park et al., 2014). The interplay between OATP2B1 and PDZK1 could be of relevance in the absorption from the lumen in the intestine, hepatocellular uptake in liver, and the reabsorption in kidney as both proteins are expressed and located in the same membrane. The relevance of the scaffold protein for in vivo pharmacokinetic of xenobiotics was described in several studies performed in Pdzk1 knockout mice. The deletion of Pdzk1 resulted in a decreased portal venous plasma concentration of the Oatp1a substrate E_1S, while the circulating plasma concentration remained comparable to that in wild-type mice (Sugiura et al., 2010). The authors explained their findings by diminished presence of Oatp1a at the apical membrane of enterocytes that reduces oral absorption. A decreased amount in the portal system is then "normalized" by decreased hepatic uptake resulting in a diminished first-pass effect (Sugiura et al., 2010). For substrates of other Pdzk1-modulated transporters, deletion of the gene locus in mice translated in significantly reduced plasma concentrations. This was reported for cephalexin (a substrate of Slc15a1; Pept1) and carnitine (a substrate of Slc22a5, Octn2) (Sugiura et al., 2008), and is in accordance with the observed reduction of both Slc transporters in the small intestine of Pdzk1$^{-/-}$ mice. Further, the protein expression of Abcg2 (Bcrp) (Shimizu et al., 2011) at the intestinal brush-border membrane, and of Abcc4 (Mrp4) (Park et al., 2014) at the apical membrane in the proximal tubules was shown to be reduced in Pdzk1 $^{-/-}$ mice. The elevated plasma concentration of cimetidine (substrate of Abcg2) and adefovir (substrate of Abcc2) supported these observations(Park et al., 2014; Shimizu et al., 2011). In the case of adefovir not only a significant change in plasma concentration, but also an alteration in AUC, clearance, and tissue concentration of the reverse transcriptase inhibitor, was observed comparing wild-type and knockout mice.

With the modulation of OATP2B1 by PDZK1, the transcriptional regulation of the scaffold protein itself moves into focus. Transcription of PDZK1 is directly regulated by HNF1α (Prestin et al., 2017a), the nuclear receptors thyroid hormones receptor α and β (Ferreira et al., 2017), and the peroxisome proliferator-activated α (PPARα) (Tachibana et al., 2008), while indirect modulation has been reported for estrogens (Kim et al., 2013; Kim et al., 2012). The change in expression of the scaffold protein would impact the stabilization/localization of OATP2B1 and therefore alter pharmacokinetics of endogenous and xenobiotic substrates of the OATP transporter.

In conclusion, we report that PDZK1 directly interacts with OATP2B1 resulting in a change of the amount of transporter in the membrane, and thereby in an increased transport function. The deletion of the C-terminally located PDZ-binding motif of OATP2B1 abolished this effect. Additionally, we reported that OATP2B1 is located in the basolateral membrane of distal tubuli and apical membrane of proximal tubuli in the kidney. These findings provide evidence that changes in PDZK1 expression may modulate the handling of OATP2B1 substrates in liver, intestine and kidney.

Conflict of interest

There is no conflict of interest to be declared by any author.

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3.5 Thyroid hormones are transport substrates and transcriptional regulators of organic anion transporting polypeptide 2B1

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Thyroid Hormones Are Transport Substrates and Transcriptional Regulators of Organic Anion Transporting Polypeptide 2B1

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ABSTRACT

Levothyroxine replacement therapy forms the cornerstone of hypothyroidism management. Variability in levothyroxine oral absorption may contribute to the well-recognized large interpatient differences in required dose. Moreover, levothyroxine-drug pharmacokinetic interactions are thought to be caused by altered oral bioavailability. Interestingly, little is known regarding the mechanisms contributing to levothyroxine absorption in the gastrointestinal tract. Here, we aimed to determine whether the intestinal drug uptake transporter organic anion transporting polypeptide 2B1 (OATP2B1) may be involved in facilitating intestinal absorption of thyroid hormones. We also explored whether thyroid hormones regulate OATP2B1 gene expression. In cultured Madin-Darby Canine Kidney II/OATP2B1 cells and in OATP2B1-transfected Caco-2 cells, thyroid hormones were found to inhibit OATP2B1-mediated uptake of estrone-3-sulfate. Competitive counter-flow experiments evaluating the influence on the cellular accumulation of estrone-3-sulfate in the steady state indicated that thyroid hormones were substrates of OATP2B1. Additional evidence that thyroid hormones were OATP2B1 substrates was provided by OATP2B1-dependent stimulation of thyroid hormone receptor activation in cell-based reporter assays. Bidirectional transport studies in intestinal Caco-2 cells showed net absorptive flux of thyroid hormones, which was attenuated by the presence of the OATP2B1 inhibitor, atorvastatin. In intestinal Caco-2 and LS180 cells, but not in liver Huh-7 or HepG2 cells, OATP2B1 expression was induced by treatment with thyroid hormones. Reporter gene assays revealed thyroid hormone receptor α–mediated transactivation of the *SLCO2B1* 1b and the *SLCO2B1* 1e promoters. We conclude that thyroid hormones are substrates and transcriptional regulators of OATP2B1. These insights provide a potential mechanistic basis for oral levothyroxine dose variability and drug interactions.

Introduction

Thyroid hormone (TH) homeostasis is essential for physiologic energy metabolism. Accordingly, alterations are linked to a variety of diseases. With a prevalence of about 4% to 5% in European and American populations, hypothyroidism (overt and subclinical) is among the most common diagnoses in endocrinology (Hollowell et al., 2002; Garmendia Madariaga et al., 2014). TH homeostasis is tightly regulated by multiple mechanisms, including those which control tissue uptake and cellular bioactivation. Thyroxine (T_4) is metabolized by intracellular iodothyronine deiodinases, which produce biologically active triiodothyronine (T_3) or inactive reverse triiodothyronine (rT_3) (Köhrle, 2007). To signal transcription, T_3 binds to intracellular TH receptors (TRs) (Mondal et al., 2016). The therapeutic management of hypothyroidism usually involves oral substitution with levothyroxine [(L-thyroxine) T_4]. However, oral absorption of L-thyroxine is highly variable and known to be affected by various factors including gastric pH or food-drug interactions (Ianiro et al., 2014). Furthermore, it is known that efficient L-thyroxine absorption from the intestinal lumen occurs in restricted segments of the gastrointestinal tract, namely, the duodenum and jejunum (Ianiro et al., 2014).

Intestinal solute transfer is mainly mediated by passive diffusion and active transport by membrane proteins. Several well-known drug-drug interactions result in reduced L-thyroxine bioavailability. For example, proton pump inhibitors alter the ionization status of L-thyroxine in the gut due to increased gastric pH, while iron and calcium supplements may form nonabsorbable chelates or complexes with THs (Centanni et al., 2006). Interestingly, a population-based

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ABBREVIATIONS: A, apical; ACN, acetonitrile; B, basal; DIO1, iodothyronine deiodinase 1; DMSO, dimethylsulfoxide; E_1S, estrone-3-sulfate; FCS, fetal calf serum; KHB, Krebs-Henseleit buffer; L-thyroxine, levothyroxine; MDCKII, Madin-Darby canine kidney II; OATP, organic anion transporting polypeptide; P_{app}, apparent permeability; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; P-gp, P-glycoprotein; QC, quality control; RPTEC, renal proximal tubular epithelial cell; rT_3, reverse triiodothyronine; RXRα, retinoid X receptor alpha; T_3, triiodothyronine; T_4, thyroxine; T_4G, thyroxine 4-O-β D glucuronide; TH, thyroid hormone; TR, thyroid hormone receptor; TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta; UHPLC-MS/MS, ultra-high-performance liquid chromatography–tandem mass spectrometry.

retrospective study observed changes in thyroid-stimulating hormone levels, a marker for TH activity, to be associated with cotreatment with statins (Irving et al., 2015). Furthermore, an acute impact of ciprofloxacin or rifampin on intestinal absorption of L-thyroxine was shown, where ciprofloxacin significantly reduced T_4 area under the plasma concentration-time curve by 39%, while rifampin significantly increased T_4 area under the plasma concentration-time curve by 25% (Goldberg et al., 2013). These later findings suggest that L-thyroxine-drug interactions may also result from mechanisms involving facilitated cellular entry or efflux (Riley et al., 2016).

With the discovery that MCT-8 (*SLC16A2*) genetic mutations cause Allan-Herndon-Dudley syndrome, a severe psychomotor retardation associated with TH dysregulation (Visser et al., 2011), it has become widely accepted that transporters are critically involved in the regulation of TH homeostasis and function (Bernal et al., 2015). Additional transporters have been reported to interact with THs, including members of the organic anion transporting polypeptides (OATPs) (van der Deure et al., 2010). Within this protein family, OATP1C1 appears to be highly active and specific for TH transport, although it is only expressed at the blood-brain barrier and in the testis (Pizzagalli et al., 2002; Sugiyama et al., 2003). In addition, the hepatic transporters OATP1B1 and OATP1B3 have been reported to mediate cellular uptake of iodothyronine sulfates (van der Deure et al., 2008), thereby contributing to hepatic TH elimination. With regard to intestinal L-thyroxine absorption, the aforementioned OATPs appear to be of minor relevance due to their expression profile. In contrast, OATP1A2 was reported to be expressed in intestine (Glaeser et al., 2007) and to recognize THs as substrates (Fujiwara et al., 2001). However, intestinal OATP1A2 expression could not be subsequently confirmed; accordingly, the transporter is mainly considered important for blood-brain transfer (Lee et al., 2005; van der Deure et al., 2010).

A leading candidate for an intestinal TH transporter is OATP2B1 (*SLCO2B1*). This sodium-independent uptake transporter is expressed in enterocytes and assumed to significantly influence oral drug absorption (Tamai et al., 2000; Kullak-Ublick et al., 2001; Drozdzik et al., 2014). While most studies supported localization of OATP2B1 at the apical membrane of the enterocyte, its polarized cellular localization is a matter of debate since basolateral sorting has recently been proposed (Kobayashi et al., 2003; Keiser et al., 2017). Despite OATP2B1 being known to mediate cellular uptake of various exogenous compounds including statins (Koenen et al., 2011), it is less certain whether this transporter is also involved in intestinal absorption of THs since there are conflicting data. Interestingly, both studies reporting data on this used a similar experimental model involving *Xenopus laevis* oocytes expressing OATP2B1 (Kullak-Ublick et al., 2001; Leuthold et al., 2009). There are several transcription start site variants of OATP2B1 (Pomari et al., 2009), which are all regulated by their own distinct promoter (Fig. 8A, 1a to 1e). Of these five variants the OATP2B1 isoform 1B (using exon 1b as the transcription start site) encodes for the original full-length protein and is the major form expressed in duodenum. All other variants that are transcriptionally controlled by different promoter regions encode for the same shortened protein lacking 22 amino acids at the N-terminus. This short variant exhibits transport of estrone-3-sulfate (E_1S) and rosuvastatin comparable to the full-length OATP2B1 isoform 1B. For the OATP2B1-1E variant, liver-enriched expression and regulation by HNF4α have recently been reported (Knauer et al., 2013). In this study, we investigated the functional and regulatory interplay of TH and the uptake transporter OATP2B1. Using cells overexpressing OATP2B1, we examined the impact of THs on cellular accumulation of the known OATP2B1-substrate E_1S. Cellular accumulation studies were supplemented by counter-flow experiments and an assessment of the influence of OATP2B1 on the transactivation of TH receptor beta (TRβ). Finally, we used the intestinal Caco-2 cell model to study transcellular TH transport as well as regulation of OATP2B1 expression.

Materials and Methods

Cell Culture. All cell lines were kept at 37°C in a humidified atmosphere supplemented with 5% CO_2. The cell lines Caco-2 (ATCC HTB37), HepG2 (ATCC HB-8065), HeLa (ATCC CCL-2), and Madin-Darby canine kidney II [(MDCKII) ATCC CRL-2936] were originally obtained from American Tissue Culture Collection (Molsheim Cedex, France). Huh-7 cells (clone JCRB0403) were purchased from the Japanese Collection of Research Bioresources (http://cellbank.nibiohn.go.jp). Primary human renal proximal tubular epithelial cells (RPTEC) were purchased from Ruwag Life science (Bettlach, Switzerland). LS180 cells were commercially obtained from Sigma-Aldrich (Buchs, Switzerland). The OATP2B1 overexpressing cell line MDCKII-OATP2B1 was generated and characterized as described elsewhere (Grube et al., 2006). Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS), and 1% GlutaMAX (Thermo Fisher Scientific, Zug, Switzerland) were used as culture medium for the Caco-2, HeLa, HepG2, Huh-7, and MDCKII cells. In the case of MDCKII-OATP2B1 the medium was supplemented with 0.25 mg/ml Hygromycin B for continuous selection. Dulbecco's modified Eagle's medium supplemented with 1% GlutaMAX, 10% FCS, and 1% nonessential amino acids was the culture medium for the LS180 cells. The RPTECs were kept in optimized Clonetics REGM renal epithelial cell growth medium supplemented as recommended by the manufacturer (Lonza, Basel, Switzerland).

In Silico Scan for Potential Thyroid Hormone Receptor Binding Sites. The previously published sequences of the *SLCO2B1* 1b promoter and the *SLCO2B1* 1e promoter (Knauer et al., 2013) were screened for potential TR binding sites using the open-access program NUBIScan version 2.0 (www.nubiscan.unibas.ch). The underlying algorithm is a joining of weighted distribution matrices of nucleotide hexamer half-sites as published by Podvinec et al. (2002). The herein used search matrix was generated based on previously described specific DNA sequence binding pattern for TRs (Ayers et al., 2014).

Immunofluorescent Staining. Cells were seeded on cover slides at a density of 75,000 cells/well in 12-well plates. After reaching 90% confluence, cells were fixed with ice-cold methanol:aceton (1:2) for 15 minutes, permeabilized with 0.2% Tween 20/phosphate-buffered saline (PBS), and then incubated with 5%-FCS-1% bovine serum albumin/PBS before adding the anti-OATP2B1-antiserum (1:100) (Grube et al., 2006) for incubation over night at 4°C. After several washes with PBS, the secondary antibody, anti-rabbit Alexa Fluor 488 (Life Technologies distributed by Thermo Fisher Scientific, Zug, Switzerland), was added for 1 hour. Prior to mounting the cells with Roti-Mount FluorCare containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclei staining, the cells were washed with PBS. Staining was detected using the Leica DMi8 Microscope (Leica Microsystems, Heerbrugg, Switzerland).

Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis. Caco-2, LS180, Huh-7, and HepG2 cells were cultured at confluence in six-well plates and then treated with 100 nM T_4 or 100 nM T_3. After 48 hours of treatment, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). The quality of the RNA was determined using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA)

and the concentration was measured by spectrophotometry (GE Nanovue Plus; GE Healthcare Life Sciences, Baie d'Urge, QC, Canada). cDNA was synthesized using the Applied Biosystems Multiscribe Reverse Transcriptase (distributed by LuBioScience, Lucern, Switzerland). Human tissue RNAs were purchased from AMS Biotechnology (Bioggio-Lugano, Switzerland) or isolated from RPTEC, Caco-2, or Huh-7 using pegGold RNA Pure (Axon Laboratory, Baden-Dättwil, Switzerland) following the manufacturer's protocol. The Life Technologies High-Capacity cDNA Reverse Transcription Kit (distributed by LuBioScience) was used for reverse transcription. The amount of mRNA was quantified using the ViiA7 Real-Time PCR System and commercially available TaqMan gene expression assays (LuBioScience) for TR beta [(TRβ), Hs00230861], TR alpha [(TRα), Hs00268470_m1], iodothyronine deiodinase 1 [(DIO1), Hs00174944_m1], and 18S ribosomal RNA [(18S rRNA), 4319413E]. The amount of *SLCO2B1* isoforms 1B and 1E was analyzed using SYBR Green PCR Master Mix (LuBioScience) and the following primers: *SLCO2B1*_1B_forward 5′-GGCTGGAGCTCACTGCAC-3′, *SLCO2B1*_1E_forward 5′-TGGGATTGAAGCTTCAGGGAG-3′, and *SLCO2B1*_1B/1E_reverse 5′-CACTGTGGAAATGGAGCTC-3′, as previously reported by Knauer et al. (2013). In the cells, DIO1 and ABCB1 were quantified using the primers DIO1_forward 5′-TTCAGCACCAGTGGCCTATT-3′, DIO1_reverse 5′-ACGACTGAGCTAGGGGGTCT-3′, P-glycoprotein (P-gp)_forward 5′-GTCCCAGGAG-CCCATCCT-3′, and P-gp_reverse 5′-CCCGGGTGTTGTCTCCAT-3′. Data were analyzed by the ΔΔCT method, where the CT values of the gene of interest were normalized to that of 18S rRNA detected in the same sample (ΔCT). The ΔCT values of each sample were referred to the mean ΔCT value of the indicated control (ΔΔCT).

Transport Studies Using MDCKII-OATP2B1 Cells. For inhibition studies and competitive counter-flow experiments, MDCKII or MDCKII-OATP2B1 cells were seeded at a density of 50,000 cells/well in 24-well plates. One day after seeding, cells were stimulated with 2.5 mM sodium-butyrate for 24 hours. For inhibition studies, cells were washed with prewarmed PBS prior to the 5-minute incubation with E_1S diluted in incubation buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM glucose, and 12.5 mM HEPES). E_1S was used at a concentration of 0.1 μM containing 100,000 dpm/well [^{3}H]-E_1S (Hartmann Analytic, Braunschweig, Germany), supplemented with the respective concentration of the tested inhibitor, namely, T_4, T_3, rT_3, and thyroxine 4-O-β-D-glucuronide (T_4G) (all obtained from Sigma-Aldrich). Atorvastatin [1 or 10 μM] was used as the control. After washing the cells three times with ice-cold PBS, cells were lysed in 200 μl 10% SDS-5 mM EDTA. Cellular accumulation of the radiolabeled substrate was determined after diluting the cell lysate in 2 ml of scintillation cocktail (Rotiszint eco plus; Carl Roth AG, Arlesheim, Switzerland) and measured using a liquid scintillation β-counter (Tricarb 2900 TR; TOPLAB, Rickenbach, Switzerland). Competitive counter-flow experiments were performed as described by Harper and Wright (2013). One day after seeding, the cells were washed once with prewarmed PBS and exposed to Krebs-Henseleit buffer [(KHB); 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4; 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, 4.7 mM KCl, pH 5.5] containing [^{3}H]-E_1S (6.06 nM/well with 100,000 dpm/well) for 30 minutes to equilibrate. After reaching steady-state conditions, the supernatant was removed and replaced by KHB containing [^{3}H]-E_1S supplemented with the test compounds, namely, E_1S (50 μM), atorvastatin (30 μM), camptothecin (1 mM), T_4 (25 μM), T_3 (10 μM), rT_3 (1 mM), or T_4G (400 μM). After 1-minute incubation the cells were washed with ice-cold PBS and the amount of radiolabel in the lysed cells was determined as described previously.

Transport Studies Using Transiently Transfected Caco-2 Cells. Caco-2 cells were seeded at a density of 75,000 cells/well in 24-well plates. One day after seeding the cells were transfected with 500 ng/well pEF6-control or OATP2B1-pEF6 using 2 μl/μg DNA jetPRIME transfection reagent (Polyplus transfection; Chemie Brunschwig AG, Basel, Switzerland), and the next day transport experiments were conducted as previously described in detail.

Cell-Based Reporter Gene Assays. To test the influence of the uptake transporter on the intracellular function of T_3 and T_4, MDCKII or MDCKII-OATP2B1 cells were transfected with a reporter gene construct containing the firefly luciferase gene under the control of the 5′ untranslated region of the iodothyronine deiodinase 1 gene (DIO1-pGL3basic) and pRL-TK encoding for *Renilla* luciferase as the transfection control. For cell-based reporter gene assays testing the transactivation of the *SLCO2B1* 1e and *SLCO2B1* 1b promoters, the respective reporter gene constructs in pGL3basic (Knauer et al., 2013) were transfected into HeLa cells. In addition to the reporter gene constructs, the cells were transfected with eukaryotic expression vectors (pEF6-V5/HIS; Invitrogen distributed by Thermo Fisher Scientific) encoding for the nuclear receptor TRβ, TRα, and/or its heterodimerization partner retinoid X receptor alpha (RXRα); in total, 25 ng pRL-TK, 250 ng of TRβ-pEF6, TRα-pEF6, and/or RXRα-pEF6 and/or pEF6 as control, and 250 ng of the pGL3basic plasmid were transfected in each well. MDCKII or MDCKII-OATP2B1 cells were seeded at a density of 40,000 cells/well in a 24-well plate, and after 24 hours the cells were transfected using 1.5 μl/μg DNA of the jetPRIME transfection reagent (Polyplus transfection). HeLa cells were seeded at a density of 50,000 cells/well in 24-well plates, and were transfected using 2.5 μl/μg DNA of the jetPRIME transfection reagent (Polyplus transfection). The next day, the cells were treated for 24 hours with 10 μM T_3 or 10 μM T_4. Subsequently, the cells were lysed in 100 μl passive lysis buffer, and then the firefly and *Renilla* luciferase activities were assessed in 20 μl of the lysate using the Dual-Luciferase Reporter Assay System (Promega, Duebendorf, Switzerland) and the infinite 200 Pro (Tecan, Maennedorf, Switzerland) according to the manufacturers' instructions. Activity of firefly luciferase was normalized to that of *Renilla*.

Western Blot Analysis. For determination of protein content, cells were lysed in 5 mM Tris-HCl (pH 7.4) supplemented with protease inhibitors (diluted 1:100; Protease Inhibitors Cocktail; Sigma-Aldrich). After three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath, the cell lysate of MDCKII cells was centrifuged at 100,000*g* (30 minutes at 4°C) for crude membrane enrichment. The resulting pellet was suspended in 5 mM Tris-HCl supplemented with protease inhibitors. For cultured Caco-2 cells, we applied the total cell lysate to immunoblotting. After adding 5x Laemmli buffer, the samples were heated for 30 minutes to 65°C prior to separation by 10% SDS-PAGE. The separated proteins were electrotransferred to a nitrocellulose membrane using a TANK blotting system (Bio-Rad, Cressier, Switzerland). Prior to incubation with the respective antibodies, anti-OATP2B1 rabbit polyclonal (Grube et al., 2006) or goat polyclonal anti-β-actin (sc-1616; Labforce, Muttenz, Switzerland), the membranes were blocked with 5% FCS in Tris-buffered saline containing 0.1% Tween 20 for at least an hour at room temperature. After several washing steps with Tris-buffered saline containing 0.1% Tween 20 the membranes were incubated with the respective horseradish peroxidase-labeled secondary antibody (dilution to 1:2000; Bio-Rad). Immobilized secondary antibody was visualized and digitalized using Western ECL Substrate (Thermo Fisher Scientific) and the ChemiDoc MP System (Bio-Rad), respectively. Analysis was performed using the Image Laboratory software (version 4.1; Bio-Rad).

Transwell Transport of Thyroid Hormones. Transepithelial solute flux studies were performed as previously published by Hubatsch et al. (2007). Briefly, Caco-2 cells were seeded at a density of 3×10^5 cells/well onto polycarbonate membranes with 0.4 μm pore size inserted in 12-well plates (Chemie Brunschwig AG). Caco-2 cells were cultivated for at least 14 days with medium change every second day, and until reaching a transepithelial electrical resistance value of at least 200 Ω/cm. Integrity of the monolayer was tested after assessment of transport using 0.1 mg/ml lucifer yellow (Sigma-Aldrich). Transport experiments were performed using KHB. In detail, after washing the cells with PBS, Caco-2 cells were preequilibrated for 20 minutes at 37°C with KHB. The apical compartment was filled with 0.5 ml of KHB, the basolateral compartment was

TABLE 1
Optimized tandem mass spectrometry parameters in electrospray ionization positive mode for analytes and the corresponding internal standards

Analyte I.S.	MRM Transition	Cone Voltage	Collision Energy
		V	*eV*
T_3	651.6 → 197.1	55	80
I.S. $^{13}C_6$ T_3	658.0 → 612.0	50	32
T_4	777.3 → 633.8	60	42
I.S. $^{13}C_6$ T_4	783.4 → 737.7	55	32

I.S., internal standard; MRM, multiple reaction monitoring.

filled with 1.5 ml of KHB, and 10 μM T_3 or 10 μM T_4 was applied to the donor compartment. Inhibition of OATP2B1 was achieved by application of 10 μM atorvastatin (USP, Basel, Switzerland) on the apical site. Accumulation in the acceptor compartment and remaining amount in the donor compartment was quantified in 200 μl aliquots taken at 30 minutes and replaced with KHB buffer. At 60 minutes a second aliquot was taken. Both apical and basolateral KHB samples were stored in glass vials (to avoid long-term nonspecific adsorption) below −65°C prior to quantification by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) as described subsequently. The apparent permeability (P_{app}) coefficient was calculated as previously described (Hubatsch et al., 2007). The determined unidirectional apparent permeability coefficients in the apical (A)-to-basal (B) or B-to-A direction were used to calculate the uptake ratio. In accordance with the commonly used efflux ratio, the uptake ratio was calculated as follows:

$$\text{uptake} - \text{ratio} = \frac{P_{app}\ (\text{A to B})}{P_{app}\ (\text{B to A})}$$

Triiodothyronine and Thyroxine Quantification by UHPLC-MS/MS. For the quantification of T_3 and T_4 in the range of 30–3000 ng/ml, specific UHPLC-MS/MS methods were developed. For both T_3 and T_4, seven calibration standards (calibrators) and three levels of quality controls (QCs) in KHB at low (QC 90 ng/ml), medium (QC 1500 ng/ml), and high (QC 2400 ng/ml) concentrations were prepared by serial dilution of the respective working solution (100 μg/ml in methanol for both T_3 and T4). The first concentration (30 ng/ml) of the calibrators was defined as the lower limit of quantification, and the highest concentration (3000 ng/ml) was defined as the upper limit of quantification. Calibrators and QCs were stored in aliquots in polypropylene tubes below −65°C until analysis. $^{13}C_6$-T_3 and $^{13}C_6$-T_4 (dissolved to 1000 ng/ml in methanol; Sigma-Aldrich) were used as internal standards for T_3 and T_4, respectively. To 20 μl of the KHB samples from the Transwell studies, 100 μl of each internal standard, 200 μl bovine serum albumin solution (60 g/l), and 700 μl of ice-cold acetonitrile (ACN) were added. The mixture was briefly vortexed, mixed for 10 minutes at 1400 rpm and room temperature, and then centrifuged for 20 minutes at 13,200 rpm and 10°C. The supernatant was transferred into a 96-deep-well plate, dried under heated nitrogen gas flow (30–50°C; Evaporex EVX-96; Apricot Designs, Monovia, CA), and reconstituted with 200 μl of injection solvent (35% eluent A and 65% eluent B; eluent A: 0.1% formic acid in water; eluent B: 0.1% formic acid in ACN) under shaking (1500 rpm) for 45 minutes at room temperature. Subsequently, each sample was transferred into a 300 μl glass insert of a high-performance liquid chromatography vial before injection into the UHPLC-MS/MS system in full-loop mode (5 μl). Quantification was performed using an UPLC HSS T3 column on an Acquity UPLC system consisting of a binary pump, an autosampler set at 10°C, and a column heater set at 45°C, which was coupled to an Acquity TQD tandem mass spectrometer (all obtained from Waters Corp., Milford, MA). The mobile phase consisted of eluents A and B. Chromatographic separation was performed at a flow rate of 0.5 ml/min with the following gradient: 0 to 1 minute, B 5%; 1–5 minutes, B 5%–100%; 5 to 6 minutes, B 100%; 6–6.01 minutes, B 100%–5%; 6.01–7 minutes, B 5%.

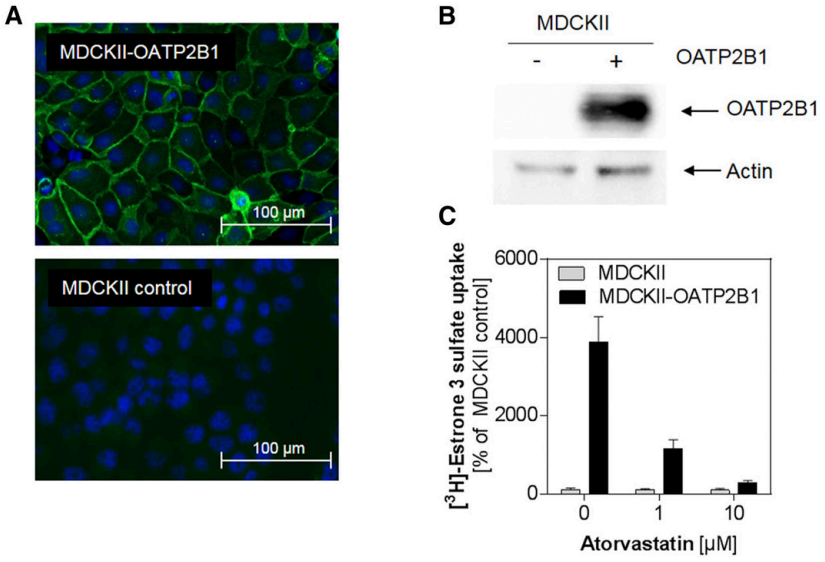

Fig. 1. Validation of OATP2B1 expression and function in stably transfected MDCKII cells. Immunofluorescent staining of MDCKII-OATP2B1 and MDCKII cells revealed localization of the transporter in the basolateral membrane of polarized MDCKII cells overexpressing the transporter. No signal was observed in MDCKII cells (A). Immunoblot analysis verified high expression when comparing MDCKII and MDCKII-OATP2B1 cells, as shown in the representative blot (B). Uptake of [^{3}H]-E_1S was significantly enhanced in the presence of the transporter, while it was reduced by co-incubation with atorvastatin (C).

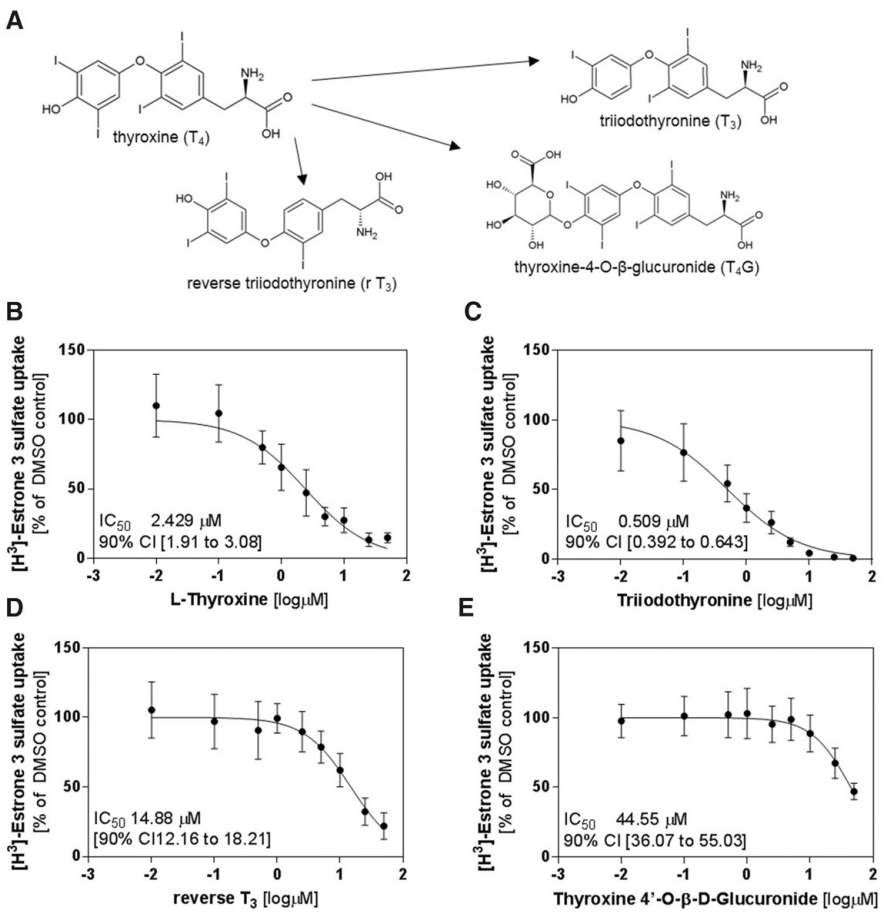

Fig. 2. Inhibition of OATP2B1-mediated uptake by TH derivatives. Uptake of [^{3}H]-E_1S in MDCKII-OATP2B1 was determined in the presence of different THs derived from T_4 (A). Cellular accumulation of E_1S was determined after incubation with increasing concentrations of T_4, (B), T_3 (C), rT_3 (D), or T_4G (E). The logarithmic inhibitor response curves were fitted to determine the IC_{50} values and the respective confidence intervals (CI). The data presented are of $n = 3$ independent replicates each in triplicate.

In addition to the aforementioned injection solvent and eluents, the weak and strong wash solvents were water-ACN (50:50, v/v) containing 0.2% trifluoroacetic acid, and ACN-isopropanol-acetone (40:40:30, v/v/v) containing 0.2% trifluoroacetic acid, respectively. The seal wash solvent consisted of a water-ACN mixture (90:10, v/v). Multiple reaction monitoring detection was performed with electrospray ionization in positive ion mode. Nitrogen was used both as desolvation and nebulization gas. Argon was used as collision gas. The tandem mass spectrometry parameters were generated using IntelliStart software (Waters Corp.) followed by manual optimization. The optimized parameters are summarized in Table 1.

Statistical Analysis. Data are presented as mean ± S.D. Data analysis was performed using GraphPad Prism software version 6.04 (GraphPad Software Inc., San Diego, CA) and Microsoft Excel (Microsoft, Redmond, WA). Values of $P \leq 0.05$ were considered as statistically significant.

Results

Influence of Thyroid Hormones on OATP2B1 Transport Activity. In a first approach, we tested the influence of T_4 and its metabolic products, namely T_3, rT_3, and T_4G (Fig. 2A) on OATP2B1-mediated uptake of the known substrate E_1S using OATP2B1 expressing MDCKII cells. Expression of OATP2B1 (isoform 1B) in the cell model was verified by immunofluorescence microscopy (Fig. 1A) and western blot analysis (Fig. 1B) showing localization of OATP2B1 at the plasma membrane of overexpressing MDCKII-OATP2B1 cells. As shown in Fig. 2, B–E, all of the tested compounds significantly inhibited OATP2B1 transport function, thereby providing the first evidence of an interaction of THs with this membrane transporter. OATP2B1 function was potently inhibited by T_4 (IC_{50} 2.43 μM, CI 2.91–3.08) and T_3 (IC_{50} 0.51 μM CI 0.39–0.64), while rT3 (IC_{50} 14.88 μM CI 12.16–18.21) and T_4G (IC_{50} 44.55 μM CI 36.07–55.03) exhibited lower inhibitory potency. Inhibition of OATP2B1 by the known OATP2B1 substrate, atorvastatin, served as a positive control of the experimental system (Fig. 1C). The influence of TH derivatives was also tested in Caco-2 cells transiently

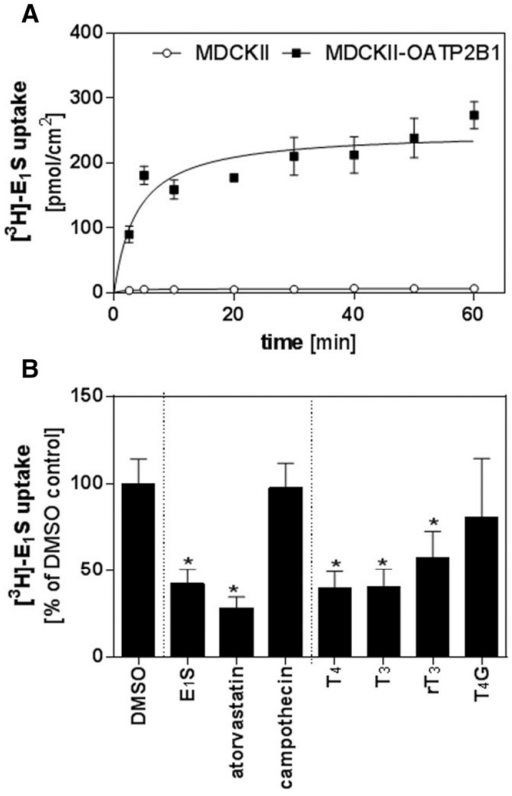

Fig. 3. Competitive counter-flow assays in MDCKII-OATP2B1 cells. Assessment of time-dependent accumulation of [^{3}H]-E_1S comparing MDCKII and MDCKII-OATP2B1 cells (A). Counter-flow experiments were conducted after reaching the steady state of E_1S at 30 minutes of incubation. The cellular amount of the radiotracer was determined after 1 minute of incubation with 50 μM E_1S, 30 μM atorvastatin, 1 mM camptothecin, 25 μM T_4, 10 μM T_3, 1 mM rT_3, or 400 μM T_4G (B). The detected amount of radiotracer was normalized to that in cells treated with the solvent control. Data are presented as mean ± S.D. ($n = 3$ independent replicates each in triplicate). For the statistical analysis, one-way analysis of variance with Dunnett's multiple comparisons test was used (*$P \leq 0.05$).

transfected with OATP2B1-pEF6 for overexpression of the transport protein. Using this cell model, we also observed inhibition of OATP2B1-mediated cellular accumulation of E_1S for T_4 (IC_{50} 4.81 μM, CI 3.65–6.34), T_3 (IC_{50} 0.97 μM CI 0.61–1.53), rT_3 (IC_{50} 16.10 μM CI 11.44–22.65), and T_4G (IC50 57.21 μM CI 38.11–85.89). Notably, the transport rate of E_1S in OATP2B1-transfected Caco-2 cells was lower than that observed for stably transfected MDCKII-OATP2B1 cells (mean transport rate ± S.D.; fmol μg protein^{-1}/min^{-1}; Caco-2-OATP2B1 vs. MDCKII-OATP2B1; 0.268 ± 0.055 vs. 1.226 ± 0.086; $n = 3$ independent replicates, each in biologic triplicate; $P < 0.05$; ordinary one-way analysis of variance with Turkey's multiple comparisons test).

Competitive Counter-Flow Transport Studies with Thyroid Hormones in OATP2B1-Expressing Cells. Subsequently, competitive counter-flow experiments were conducted to determine whether the observed inhibition is due to transport of the tested TH derivative. Counter-flow was assessed in MDCKII-OATP2B1 cells at equilibrium, which was reached after 30 minutes of incubation with E_1S (compare Fig. 3A). An examination of the remaining amount of radiolabeled [^{3}H]-E_1S in cells exposed to T_4 (25 μM), T_3 (10 μM), and rT_3 (1 mM) revealed significantly lower levels of the radiolabel compared with cells exposed to dimethylsulfoxide (DMSO) vehicle control (mean percentage of DMSO control ± S.D.; T_4: 39.41% ± 9.93%, T_3: 40.34% ± 10.37%; rT_3: 57.20% ± 15.37%; one-way analysis of variance with Dunnett's multiple comparisons test; $P < 0.05$), suggesting that these compounds are OATP2B1 substrates. In this assay, the known OATP2B1 substrates atorvastatin (30 μM; 28.33% ± 6.17%) and E_1S (50 μM; 42.31% ± 8.26%) expectedly reduced intracellular E_1S after addition in the steady state. Lastly, T_4G (400 μM; 80.70% ± 33.70%) and camptothecin (97.71% ± 14.07%) did not affect cellular E_1S equilibrium (Fig. 3B), indicating that these compounds were not substrates of OATP2B1.

Influence of OATP2B1 Transport Function on Intracellular Thyroid Hormone Effects. To provide additional evidence for OATP2B1 transport of THs, we tested the impact of OATP2B1 expression on intracellular TH signaling. MDCKII or MDCKII-OATP2B1 cells were used for cell-based reporter gene assays that examined transactivation of the human DIO1 promoter (see the illustration below the graphs in Fig. 4). With overexpression of the OATP2B1 transporter, transactivation of the DIO1 promoter was significantly enhanced by both T_3 and T_4 with cotransfection of TRβ with or without RXRα (Fig. 4, A and B). Thyroid hormones did not activate the DIO1 promoter in both cell lines transfected with just RXRα (Fig. 4C); indicating the signal enhancement was TRβ dependent. It is notable that the transfection efficacy in MDCKII was much lower than that of HepG2 or HeLa cells in cell-based reporter gene assays conducted in our laboratory. This, in part, explains the lower transactivation of the DIO1 promoter by about 3-fold, since we usually see a transactivation of about 5- to 6-fold compared with vector control in experiments with cells better accessible by transfection.

Expression of OATP2B1 and TRβ in Human Tissues and Cellular Models. Different tissues and commonly used cellular models of those organs were analyzed by real-time PCR to determine endogenous expression of the transporter OATP2B1 isoform 1B, OATP2B1 isoform 1E, TRβ, TRα, and DIO1. The DIO1 was only observed in liver kidney, and in Caco-2, HepG2, Huh-7, and RPTECs but not in LS180 cells (mean expression ± S.D., relative to liver, Caco-2 vs. LS180 vs. Huh-7 vs. HepG2 vs. RPTEC, DIOI: 0.779 ± 0.313 vs. not detected vs. 0.038 ± 0.003 vs. 0.162 ± 0.027 vs. 0.006 ± 0.006, data not shown). As shown in Fig. 5, A and B, the mRNA of TRα or TRβ was observed in all tissues studied. Examination of the mRNA expression of the OATP2B1 isoforms verified the previous observation of isoform 1B as enriched in the intestine, while isoform 1E (Fig. 5E) was predominantly expressed in liver (Fig. 5F) (Knauer et al., 2013). For all genes examined in different cell models, the highest expression levels were observed in intestinal Caco-2 cells compared with LS180, Huh-7, HepG2, and RPTEC (mean expression ± S.D., relative to liver, Caco-2 vs. LS180 vs. Huh-7 vs. HepG2 vs. RPTEC, TRα: 36.266 ± 7.289 vs. 28.116 ± 6.395 vs. 12.942 ± 3.582 vs. 2.815 ± 0.374 vs. 7.531 ± 1.686; TRβ: 4.521 ± 1.224 vs. 4.321 ± 0.372 vs. 4.614 ± 0.315 vs. 1.022 ± 0.162 vs. 1.384 ± 0.157, OATP2B1-1B: 149.599 ± 66.648 vs. not detected vs. 14.071 ± 4.627 vs. 5.358 ± 0.956 vs. 0.145 ± 0.074 and

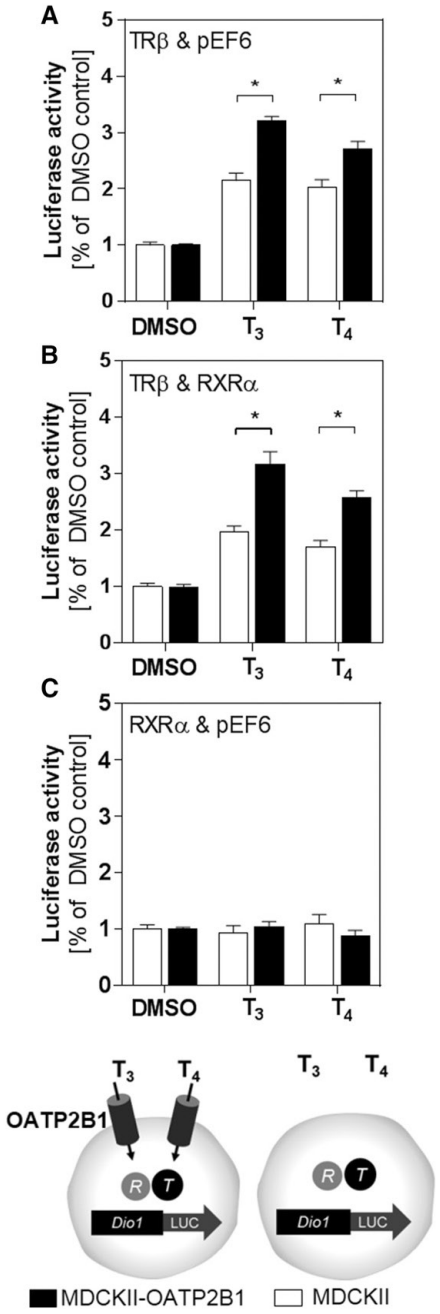

Fig. 4. Influence of OATP2B1 expression on transactivation of the DIO1 promoter. Transactivation of the DIO1 promoter was determined by cell-based dual luciferase assays comparing MDCKII with MDCKII-OATP2B1 cells (see the illustration below the graphs). Luciferase activity was assessed after treatment with T_3 or T_4. MDCKII or MDCKII-OATP2B1 cells were transfected with DIO1-pGL3basic and eukaryotic expression vectors encoding for TRβ (A), RXRα (B) or both (C). Data are presented as mean ± S.D. (n = 3 independent replicates each in triplicate). For the statistical analysis, two-way analysis of variance with Sidak's multiple comparisons test was used (*$P \leq 0.05$).

OATP2B1-1E: 9.278 ± 2.381 vs. 3.553 ± 0.334 vs. 0.297 ± 0.073 vs. 0.222 ± 0.083 vs. 0.002 ± 0.001; Fig. 5, C, D, G, and H), thereby supporting further use of Caco-2 cells for in vitro studies on the interactions of OATP2B1 and THs.

Quantification of Transcellular Transport of Thyroxine and Triiodothyronine in Caco-2 Cells. Transcellular flux of T_4 or T_3 was assessed in differentiated Caco-2 cells cultured in Transwells. The amount of T_4 and T_3 in the apical or basal compartment was quantified by UHPLC-MS/MS. Solute flux studies in the A-to-B direction revealed that T_4 exhibits intermediate permeability, while T_3 shows low permeability since the P_{app} value was below 3.3×10^{-6} cm/s [mean $P_{app\ (A\text{-}B)}$ ± S.D. (cm/s), T_4: $3.70 \times 10^{-6} \pm 0.17 \times 10^{-6}$; T_3: $2.75 \times 10^{-6} \pm 7.08 \times 10^{-9}$]. Lower permeability was observed when examining transcellular transfer of THs in the B-to-A direction [mean $P_{app\ (B\text{-}A)}$ ± S.D. (cm/s), T_4: $2.08 \times 10^{-6} \pm 1.55 \times 10^{-9}$; T_3: $0.893 \times 10^{-6} \pm 2.51 \times 10^{-9}$]. Similar results were obtained with T_4 and T_3 permeability after 60 minutes of incubation using the same system in the A-to-B direction [mean $P_{app\ (A\text{-}B)}$ ± S.D. (cm/s); T_4: $2.75 \times 10^{-6} \pm 0.57 \times 10^{-6}$; T_3: $3.36 \times 10^{-6} \pm 0.77 \times 10^{-6}$, data not shown] or B-to-A direction [mean $P_{app\ (A\text{-}B)}$ ± S.D. (cm/s); T_4: $2.34 \times 10^{-6} \pm 0.32 \times 10^{-6}$; T_3: $1.74 \times 10^{-6} \pm 0.36 \times 10^{-6}$]. However, calculating the uptake ratio—which was conducted in a similar manner as the commonly used efflux ratio, but by dividing $P_{app\ (A\text{-}B)}$ by $P_{app\ (B\text{-}A)}$—revealed factors of 1.779 ± 0.130 and 3.203 ± 0.909 for T_4 and T_3, respectively; highlighting that there is a component in cellular transfer that enhances A-to-B flux, despite that low permeability observed. Addition of atorvastatin (10 μM) as a known inhibitor of OATP2B1 in the bidirectional permeability assessment significantly changed the uptake ratios ($P_{app\ (A\text{-}B)}/P_{app\ (B\text{-}A)}$) for T_4 and T_3 (Fig. 6). These results indicate the presence of atorvastatin-sensitive, TH transporters in Caco-2 cells.

Transcriptional Regulation of OATP2B1 by Thyroid Hormones. Furthermore, we examined whether T_4 (100 nM) or T_3 (100 nM) treatment influences the expression of OATP2B1 variants in Caco-2 or Huh-7 cells. Both OATP2B1 1B and 1E variants are expressed in Caco-2 and Huh-7 cells (Knauer et al., 2013). We found that T_3 and T_4 treatments increased the mRNA expression of the intestinal OATP2B1 1B variant by 10-fold (mean % of DMSO control ± S.E.M., 1008% ± 240% of control, $P < 0.05$), and 4.8-fold (480% ± 140% of control, $P < 0.05$), respectively, in Caco-2 cells (Fig. 7A). However, the THs did not appreciably increase the OATP2B1 1B mRNA expression in Huh-7 cells (Fig. 7D). The liver-enriched OATP2B1 1E variant mRNA expression was also induced 5.5-fold (549% ± 118% of control, $P < 0.05$) by T_3 and 2.7-fold (265% ± 69% of control, $P < 0.05$) by T_4 in Caco-2 cells (Fig. 7B), but again not in Huh-7 cells (Fig. 7E). For controls, we examined the expression of DIO1 and ABCB1 (P-gp); two well-recognized TR target genes. As expected, both T_3 and T_4 treatments induced the expression of DIO1 in both Caco-2 (Fig. 7C) and Huh-7 cells (Fig. 7F). Induction was also observed for the expression of ABCB1 in Caco-2 (mean % of DMSO control ± S.D., T_3: 9100% ± 8366%; T_4: 2054% ± 507.8%; n = 5, Kuskal-Wallis test, $P < 0.05$) and in Huh-7 cells (T_3: 148.2% ± 52.6%; T_4:

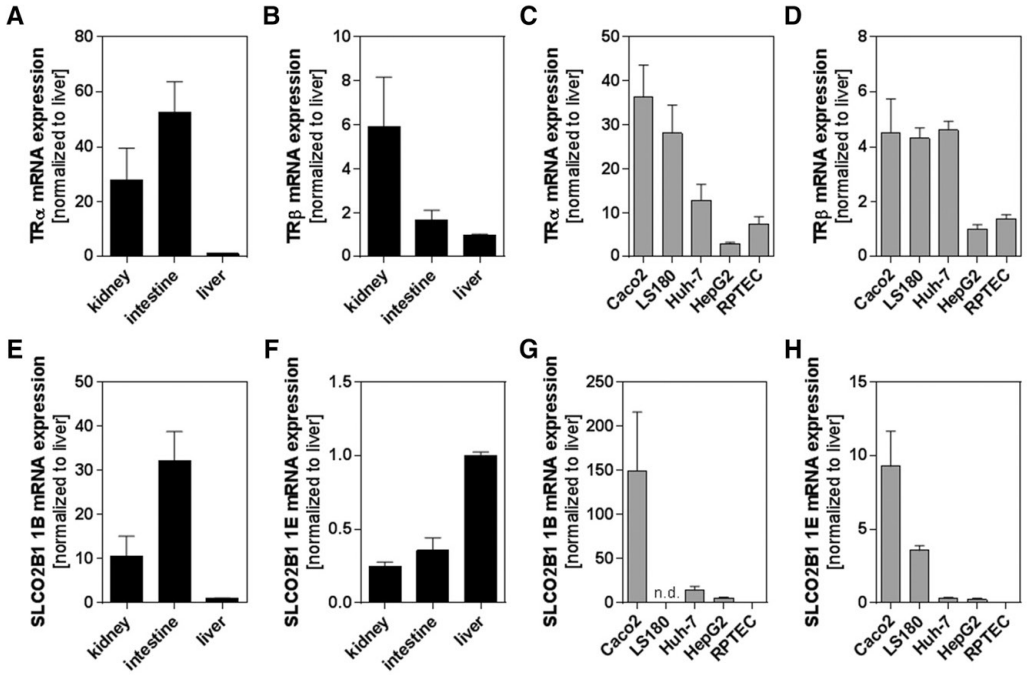

Fig. 5. Gene expression analysis in tissues and commonly used cellular models. Expression of TRα and TRβ was quantified by real-time PCR in different human tissues (A and B) and corresponding cellular models (C and D) using specific TaqMan gene expression assays. In addition, mRNA amount of two isoforms of the OATP2B1 was assessed. Both isoform 1B and isoform 1E (*SLCO2B1* 1B and 1E) were quantified with SYBRgreen assays (E–H). Relative expression of each gene was calculated according to the $\Delta\Delta$Ct method. Data are presented as mean ± S.D., $n = 3$ independent replicates each in triplicate.

161.1% ± 63.87%; $n = 5$, Kuskal-Wallis test, $P < 0.05$) (data not shown). Similar results were observed when testing the influence of THs on OATP2B1 protein expression in differentiated Caco-2 cells cultivated for 14 days prior to TH exposure. As shown in Fig. 7, G and H, expression of OATP2B1 was significantly increased by 2.6-fold in the presence of T_3 and by 2.9-fold in the presence of T_4. We additionally examined TH-mediated regulation of OATP2B1 expression in other intestinal and liver cell lines. With intestinal LS180 cells, there was significant increment in the OATP2B1 isoform 1E mRNA expression after exposure to T_3 (mean expression % of DMSO control ± S.D.; DMSO vs. T_3 vs. T_4; 117.92 ± 12.99 vs. 294.07 ± 41.46 vs. 244.36 ± 156.71; $n = 3$ independent replicates each in triplicate; $P < 0.05$; Kruskal-Wallis test with Dunn's multiple comparisons test). In LS180 cells, the amount of ABCB1 mRNA was also significantly increased by T_3 treatment (DMSO vs. T3 vs. T4; 121.93 ± 16.95 vs. 1286.14 ± 256.45 vs. 738.32 ± 400.22; $P < 0.05$). However, for both genes we did not observe changes in expression after exposure of LS180 cells to T_4. It is notable that the mRNA expressions of the OATP2B1 1B variant and that of DIO1 were not detected in LS180 cells. In HepG2 cells we tested the influence of T_3 on DIO1, ABCB1, and the OATP2B1 variants, and observed no significant change in mRNA expression of any gene (mean expression % of DMSO control ± S.D.; DMSO vs. T_3 DIO1: 105.01 ± 2.81 vs. 155.71 ± 19.93; ABCB1: 105.99 ± 2.48 vs. 89.96 ± 15.25; OATP2B1 1E: 103.81 ± 1.64 vs. 144.9 ± 10.11; OATP2B1 1B 104.81 ± 2.22 vs. 139.92 ± 5.60; $n = 3$ independent replicates each in triplicate; $P > 0.05$ $n = 3$ independent replicates each in triplicate; Mann-Whitney test). Taken together, these results demonstrate that THs are positive regulators of OATP2B1 gene expression in a cell type–dependent manner.

OATP2B1 Promoter Analysis and Cell-Based Reporter Gene Assays. Transcriptional regulation by THs is mediated by TRs. These nuclear receptors bind to DNA response elements in the promoter region of their target genes. We performed an in silico analysis to search for the TR DNA-binding motifs DR4, ER6, and DR1 in the *SLCO2B1* 1b or *SLCO2B1* 1e promoters (Knauer et al., 2013). As summarized in Table 2 and shown in Fig. 8A, multiple potential response elements for TRs were identified in the sequences analyzed. In detail, the ER6 motif (AGTCCTcagtccAGGAAA) in positions −899 to −882 exhibited the highest rank in the analysis of the *SLCO2B1* 1b promoter sequence, while the previously reported DR1 motif (AGGGCAaAGTCCA in positions −17 to −4), had the highest score in the *SLCO2B1* 1e promoter. Considering the differential tissue/cell expression of TRα and cell-type specific induction of OATP2B1 by THs, we examined whether the SLCO2B1 1b and SLCO2B1 1 e promoters were activated in the presence of TRα and its activating ligand T_3 in cell-based luciferase assays. The TR-sensitive DIO1 promoter served as the control in these experiments. We observed significantly enhanced luciferase activity after treatment with T_3 in HeLa cells transfected with the *SLCO2B1* 1b promoter (mean luciferase activity fold of pGL3b control ± S.D.; DMSO vs. T_3; 1.364 ± 0.141 vs.

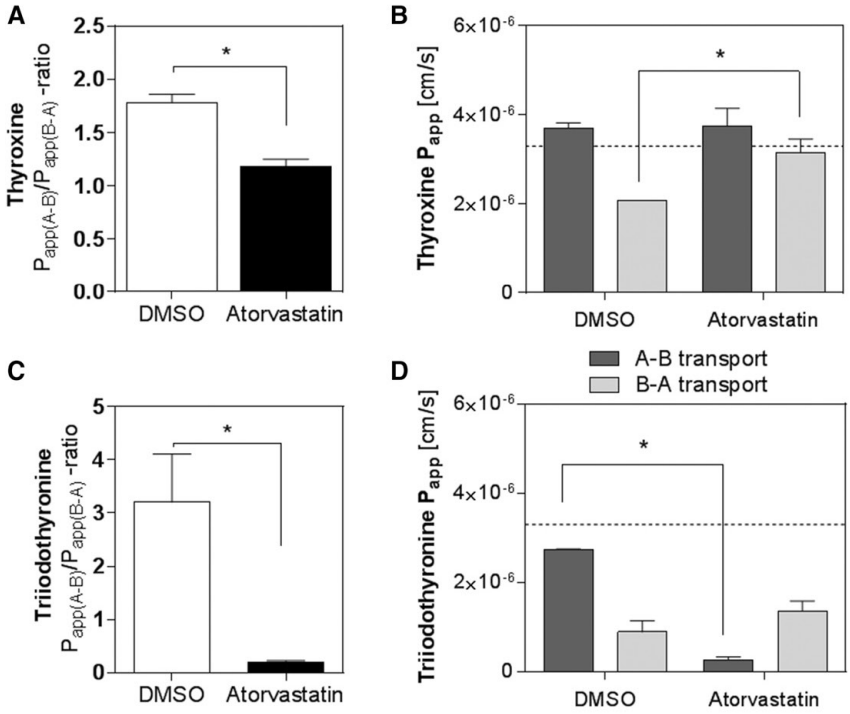

Fig. 6. Transcellular flux of THs and localization of OATP2B1 in cultured Caco-2 cells. Caco-2 cells cultivated for 14 days in a Transwell system were used to assess apical-to-basolateral (A-B) or basolateral-to-apical (B-A) flux of T_4, or T_3 in presence of solvent control (DMSO), or atorvastatin (10 μM). The unidirectional P_{app} coefficients for T_4 (B) and T_3 (D) were used to calculate the uptake ratios [P_{app} (A-B)/P_{app} (B-A)] for T_4 (A) and T_3 (C). Data are presented as mean ± S.D. (n = 3 independent replicates). For statistical analysis, Sidak's multiple comparisons test was used (*$P \leq 0.05$).

2.268 ± 0.444; $P < 0.05$ student's t test, Fig. 8B). Similarly, T_3 treatment enhanced the *SLCO2B1* 1e promoter luciferase activity (1.197 ± 0.142 vs. 2.260 ± 0.485; $P < 0.05$ student's t test, Fig. 8C). These results demonstrate that both SLCO2B1 1b and SLCO2B1 1e promoters are transactivated by TRα.

Discussion

We report interaction of THs with the function and expression of OATP2B1. This ubiquitously expressed transporter, with suspected function in the intestinal absorption of drugs, was inhibited by all TH derivatives tested in OATP2B1-expressing cells. Furthermore, counter-flow experiments assessing the influence of TH derivatives on the cellular equilibrium of E_1S at steady state suggested that T_3, T_4 and rT_3 are not only inhibitors but also substrates of OATP2B1. This notion was further supported by findings showing that the presence of OATP2B1 significantly enhanced intracellular TH signaling as observed in cell-based reporter gene assays comparing MDCKII and MDCKII-OATP2B1 cells. The presence of an active transport component in the transcellular transport of T_3 and T_4 was supported by results of transcellular flux experiments in the intestinal cell line Caco-2. Indeed, we observed enhanced permeability in the A-to-B direction for both T_4 and T_3, resulting in uptake ratios ($P_{app\ (A\text{-}B)}/P_{app\ (B\text{-}A)}$) of about 1.8 and about 3.2 for T_4 and T_3, respectively. Atorvastatin, which is a known substrate and competitive inhibitor of OATP2B1 (Grube et al., 2006), significantly reduced this ratio for T_3 and T_4. The observed reduction in the TH uptake ratio by atorvastatin could certainly be interpreted in terms of reduced uptake mediated by apically localized OATP2B1, but it should also be noted that multiple transporters are expressed in enterocytes and Caco-2 cells, of relevance being the efflux transporter ABCB1 (P-gp), which has previously been reported to also transport T_3 (Mitchell et al., 2005). Moreover, atorvastatin is not a specific OATP2B1 inhibitor, and thus also interacts with several efflux transporters including the aforementioned ABCB1 (Chen et al., 2005). Despite that, we observed reduction in the net absorption of THs in the presence of atorvastatin in Caco-2 cells, even if it is likely that there has been reduced apical TH efflux by ABCB1 in the presence of atorvastatin.

As previously mentioned, localization of OATP2B1 in enterocytes is a matter of an ongoing debate, which was sparked by recent experiments by Kaiser et al. (2017), indicating that OATP2B1 is localized in the basolateral membrane of enterocytes. This localization would be expected to result in an increase in the uptake ratio of OATP2B1 substrates in the presence of an inhibitor specific to this transporter, which has not been observed in the herein reported experiments. Even if we are not able to dissect the

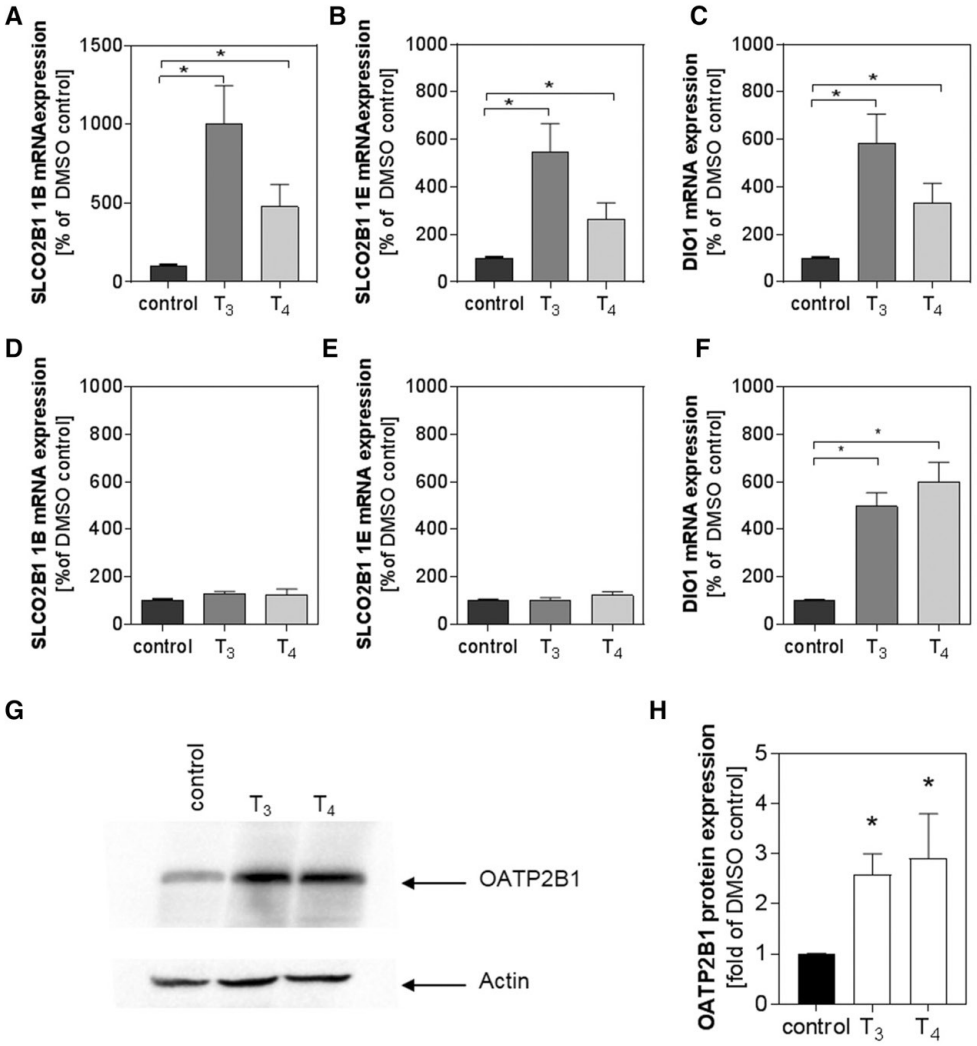

Fig. 7. Influence of THs on expression of OATP2B1. Expression of *SLCO2B1* isoform 1B (A) *SLCO2B1* isoform 1E (B) and deiodinase type 1 (C) mRNA was quantified by real-time PCR after 48 hours of treatment of differentiated Caco-2 cells with T_3 (100 nM) or L-thyroxine (T_4, 100 nM). Amount of *SLCO2B1* isoform 1B (D), *SLCO2B1* isoform 1E (E), and deiodinase type 1 (F) mRNA was quantified by real-time PCR in Huh-7 cells treated with T_3 (100 nM) or T_4 (100 nM). Expression data were calculated by the ΔΔ-Ct method, where expression was related to that of a housekeeping gene and then normalized to the solvent control. Data are presented as mean ± S.E.M. of $n = 5$ experiments. Gene expression was analyzed with a Kruskal-Wallis test with Dunn's multiple comparisons test. OATP2B1 protein expression was assessed by western blot analysis [representative result, (G)]. Actin served as the loading control (H). Data obtained by densitometry of $n = 3$ experiments are presented as mean ± S.D., and comparisons were analyzed by one-way analysis of variance with Dunnett's multiple comparisons test (E) (*$P < 0.05$).

contribution of each Caco-2 transporter to TH fluxes since atorvastatin is a potent inhibitor of OATP2B1, but not specific for this transporter, our observation, which showed a reduction in the ratio, would be in agreement with findings by others showing expression of OATP2B1 in the apical membrane of enterocytes and Caco-2 cells (Tamai et al., 2001; Kobayashi et al., 2003).

Our data demonstrated inhibition of OATP2B1 function by T_3 and T_4 with in vitro IC_{50} values in the micromolar range. Whether this inhibition is of clinical relevance with respect to TH being a perpetrator of pharmacokinetic drug interactions, especially in other tissues than intestine, is uncertain. However, normal T_4 plasma concentrations range from 0.59 to 1.54 μM. Furthermore, T_4 is significantly bound to serum proteins resulting in free T_4 concentrations of 0.09–0.24 nM. Considering these low physiologic free plasma concentrations, it seems unlikely that T_4 would alter the tissue distribution/elimination of drugs through inhibition of OATP2B1. Similar

TABLE 2

Summary of potential TR response elements in the SLCO2B1 1b and SLCO2B1 1e promoter region

Analysis was performed using the publically available NUBIscan software. Response element marked with † exhibited the highest score in the in silico analysis for this particular motif. Response element highlighted in bold had the highest score in the analysis of the respective promoter fragment. Response element marked with * was previously reported to be the most likely HNF4α binding site, resulting in constitutive activity of the promoter in cells, where this transcription factor is present (Knauer et al., 2013).

Motif	Sequence	Position
SLCO2B1-1b promoter		
DR4-III†	GGGTCTgatcAGGTCA	−1756 to −1741
DR4-II	AGGACAgagaAGCCAA	−1078 to −1063
ER6-II	TATCCTgggtggAGGGAA	−1362 to −1345
ER6-I†	**AGTCCTcagtccAGGAAA**	**−899 to −882**
DR4-I	AGGCTGgaaaAGGACA	−405 to −390
SLCO2B1-1e promoter		
DR4-IV†	AGCACAtctgAGGCAA	−2170 to −2186
DR1-V	AGAGCAaGGGCCA	−2136 to −2149
ER6-I	TGACCTatttcaGGGTGA	−1241 to −1223
DR4-III	AGGTGGgagaAGGTCA	−1222 to −1206
DR1-IV	AGGTCTgAGGCCT	−996 to 983
DR1-III	AGGACAgATCTCA	−954 to −941
DR1-II	AGCCCAcAGGAAA	−167 to −155
DR4-II	AGGGAAgcacTGGCC	−55 to −40
DR4-1	TGGGCAggggAGGGAA	−46 to −30
DR1-I†*	**AGGGCAaAGTCCA**	**−17 to −4**

considerations could be taken for T_3, whose physiologic plasma total concentrations range from 0.01 to 0.03 μM.

At the clinical level, little is known regarding the role of OATP2B1 in the intestinal absorption of L-thyroxine. A clinical study was conducted by Lilja et al. (2005), motivated by a case in which a patient experienced significant changes in L-thyroxine efficacy after grapefruit juice ingestion. Grapefruit juice is known to inhibit intestinal OATP2B1 (Satoh et al., 2005). The investigators found that grapefruit juice slightly reduced the L-thyroxine area under the plasma concentration-time curve by 9% within 0–6 hours, which was concluded to be clinically insignificant. While this finding

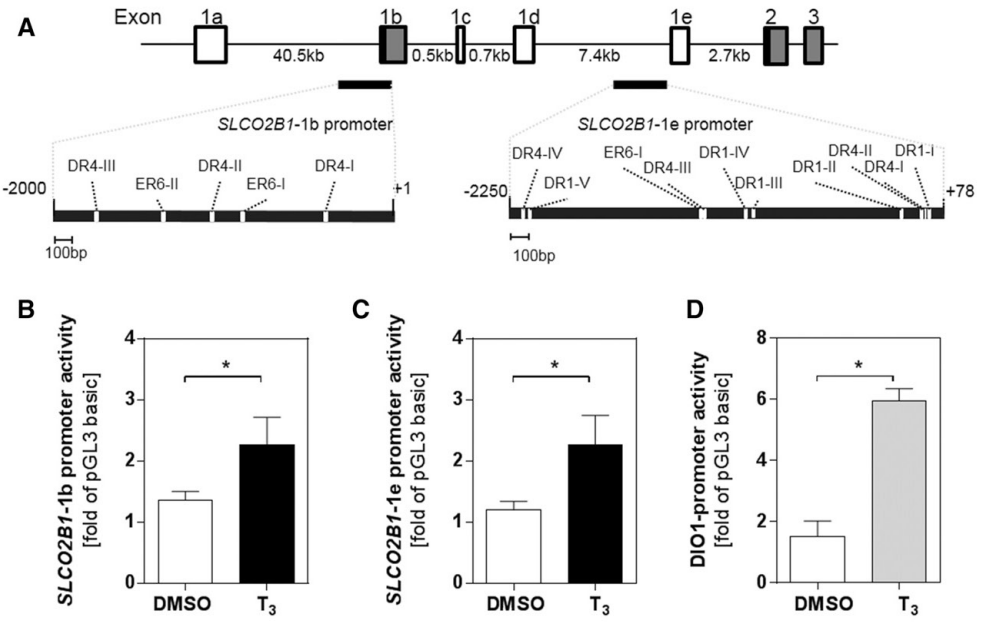

Fig. 8. TRα-mediated transactivation of the SLCO2B1-1b or SLCO2B1-1e promoter. Arrangement of the respective region of the *SLCO2B1* gene locus depicting the different exons 1a to 1e. For the transcriptional start site variants SLCO2B1-1B and 1E, details on the localization of potential thyroid hormone receptor response elements in the respective promoter region are shown (A). Cell-based reporter gene assays were performed in HeLa cells transiently transfected with the SLCO2B1-1b (B), SLCO2B1-1e (C), or the Dio1 (D) promoter and treated with T_3 (10 μM) or solvent control. Data are shown as the mean luciferase activity fold of pGL3-basic ($n = 3$ independent replicates each in triplicate); *, <0.05 Student's t test.

argues against the role of OATP2B1 in the oral absorption of L-thyroxine, it is notable that inhibition of OATP2B1 by grapefruit juice may be drug specific since the transporter possesses two binding sites for substrates and inhibitors (Shirasaka et al., 2012, 2014). Characterization of L-thyroxine binding to the high- and low-affinity binding sites and modulation by grapefruit juice remains to be determined. Another explanation for the lack of a significant pharmacokinetic interaction in the aforementioned study may be that it was conducted with healthy volunteers, while in the clinical case report the patient had hypothyroidism. The possibility that hypothyroidism may influence the magnitude of the juice/L-thyroxine interaction is supported by our finding that OATP2B1 expression is regulated by THs.

We showed upregulation of OATP2B1 mRNA and protein expression in Caco-2 cells by THs, suggesting that TH status influences the expression and activity of the intestinal uptake transporter. A similar transcriptional regulation was observed when assessed in LS180 cells, an additional intestinal cell model previously used to show induction of ABCB1 after exposure to THs (Mitin et al., 2004). Interestingly, when examined in Huh-7 and HepG2 cells, models for hepatocytes, THs did not affect OATP2B1 expression, pointing to cell model–specific regulation. Expression of TRβ was observed in all cell models used in our study, suggesting that differential expression of this receptor is not the reason underlying the cell-specific effect. In contrast, quantification of TRα revealed a lower amount of this nuclear receptor in hepatic cell models, suggesting that this nuclear receptor may be involved in the regulation of OATP2B1 expression observed in the intestinal cells. Two N-terminal protein variants of OATP2B1 (variants 1B and 1E) have been described; these variants are assumed to be transcriptionally regulated by different promoter regions since their transcriptional start sites differ. In accordance are findings showing that the liver-enriched *SLCO2B1* 1E, but not the ubiquitously expressed *SLCO2B1* 1B transcriptional start site variant, is regulated by the transcription factor HNF4α (Knauer et al., 2013). However, with respect to regulation by TRα we observed significant transactivation of both *SLCO2B1* promoters. Considering that multiple potential binding sites for TRs were predicted in the promoter sequences of *SLCO2B1*-1b and *SLCO2B1*-1e and that TRα is the predominant nuclear receptor in intestine (Sirakov and Plateroti, 2011), differences in TRα expression levels may be the mechanism underlying the observed differences in transcriptional regulation in hepatic (Huh-7 and HepG2) and intestinal (Caco-2 and LS180) cells.

There is evidence that TH status modulates expression and function of other drug transporters in the intestinal wall. For example, the efflux transporter ABCB1 that limits intestinal drug absorption is regulated by THs (Mitin et al., 2004). Indeed, the required doses of the ABCB1 substrate digoxin used to treat cardiac failure and to control heart rate differ among patients suffering from hyper- or hypothyroidism compared with patients with normal thyroid function. Specifically, higher and lower digoxin maintenance doses are needed with hyperthyroidism and hypothyroidism, respectively (Burk et al., 2010). It is notable that digoxin is not a substrate of OATP2B1 (Taub et al., 2011). Another study by Jin et al. (2005) showed that the trough plasma concentrations of the ABCB1 substrate cyclosporin A were lower in patients taking L-thyroxine. Increased expression of abcb1a/abcb1b in mice after long-term treatment with L-thyroxine was found to explain this clinical drug-drug interaction. Lastly, Siegmund et al. (2002) reported induction of intestinal ABCB1 in humans treated with L-thyroxine. However, this induction of ABCB1 resulted only in minor, nonclinically relevant changes in the pharmacokinetics of talinolol, an ABCB1 substrate (Siegmund et al., 2002). It is interesting that talinolol is also a substrate of the uptake transporters OATP2B1 and OATP1A2 (Shirasaka et al., 2010), despite that it is not anionic. It is possible that TH-mediated upregulation of the intestinal efflux mechanisms could be counteracted by the concomitant upregulation of OATP2B1, leading to minimal pharmacokinetic consequences for talinolol.

In conclusion, we report that T_3 and T_4 are substrates of OATP2B1, indicating a novel mechanism for regulation of TH homeostasis by influencing hormone distribution and absorption during replacement therapy. In addition, we showed upregulation of OATP2B1 by THs, which may be of relevance for intestinal absorption of substrate drugs with narrow therapeutic index.

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Authorship Contributions

Participated in research design: Meyer zu Schwabedissen, Ferreira, Schaefer, Oufir, Tirona.

Conducted experiments: Meyer zu Schwabedissen, Ferreira, Schaefer, Oufir, Seibert, Tirona.

Contributed new reagents or analytic tools: Meyer zu Schwabedissen, Hamburger, Tirona.

Performed data analysis: Meyer zu Schwabedissen, Ferreira, Schaefer, Oufir, Tirona.

Wrote or contributed to the writing of the manuscript: Meyer zu Schwabedissen, Ferreira, Schaefer, Oufir, Hamburger, Tirona.

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4 Conclusions

The discovery of proteins participating in absorption, distribution, metabolism and elimination of drugs and the investigation of their mechanism of action led to a better understanding of pharmacokinetics and pharmacodynamics [1]. Drug transporters are highly related to the kinetic of their substrates facilitating their membrane crossing. Nevertheless, proteins do not act as single entity but are often part of a network influenced by other proteins. PDZK1, a PDZ domain containing scaffold protein, is one of those proteins directly modulating membrane transporters and receptors [75, 91]. Hence, PDZK1 exerts an indirect impact on the substrates of their targeted proteins by modulating their cell surface expression and activity [82-84, 89]. Many of the proteins modulated by PDZK1 are members of the SLC and ABC superfamily, two transporter families linked to drug pharmacokinetic [78, 86].

As a result, the transcriptional regulation of PDZK1 itself came into focus of the pharmacological research. Genome wide association studies (GWAS) showed a link between the uric acid levels and single nucleotide polymorphism (SNPs) located in the promoter of PDZK1(rs1967017 -4017 bp, rs1471633 -3923 bp, rs12129861 -1976 bp) [114-117]. This association of PDZK1 with the uric acid levels and several studies showing the modulation of urate transporters by the scaffold protein generated the idea of the "urate transportosome" [86, 89, 105, 118, 119] . In this context we reported that upon activation the thyroid hormone receptor beta (THRβ) increases the mRNA and protein expression of the PDZK1 and that the binding site of THRβ included the SNP rs1967017 (-4017 bp) linked to the urate acid homoeostasis. The exchange of thymine to cytosine (SNP rs1967017) reduced the promoter activity by nearly 30% of the scaffold protein. Interestingly, the experiments were performed in Caco2 cells, a widely accepted cellular model for intestinal transport, expanding the regulation of PDZK1 by THRβ to other tissues than the kidney or the liver where THRβ is assumed to be predominant [120, 121]. In addition, stabilized transporters as a result of an increased expression of PDZK1 and therefore augmented in- and efflux could be hypothesized as one of the mechanisms behind the hypermetabolic effect by excess of thyroid hormones.

A transporter of SLCO family that interacts with PDZK1 is the Organic anion transporting polypeptide 2B1 (OATP2B1). OATP2B1 inherits a PDZ binding motif at C-terminus and therefore an interplay between the membrane transporter and PDZ proteins seemed likely. We investigated impact of PDZK1 on OATP2B1 showing an increased membrane presence of the membrane transporter resulting in emerged maximal transport velocity (V_{max}) for the prototypical substrate estrone 3-sulfate (E_1S). Also, PDZK1 binds directly to OATP2B1 and the impact of the scaffold protein could be abolished by deletion of the PDZ binding motif at the C-terminus of membrane transporter. Furthermore, we located in the kidney OATP2B1 at apical membrane of proximal tubulus and at the basolateral membrane of distal tubulus.

PDZK1 and OATP2B1 share more than just the interaction between them also both proteins are linked to the thyroid homoeostasis. PDZK1 is regulated by the thyroid hormone receptors and *Leuthold et al.* showed that thyroxin (T4) is a substrate of OATP2B1 [24]. The questions remained whether OATP2B1 transports other thyroid hormones than T4 and if it is regulated like the scaffold protein by the thyroid hormones. Indeed, triiodothyronine (T3) is a substrate of OATP2B1 and the mRNA as well as the protein expression are increased in Caco2 by the presence of T3

and T4. In combination with the direct regulation of PDZK1 by thyroid hormones receptors and the interaction of the scaffold protein with OATP2B1 may indicate that both proteins are part of the thyroid homoeostasis.

The first described transcriptional regulator of PDZK1 was the peroxisome proliferator-activated receptor α (PPARα) [96]. PPARα exhibits an important role in the lipid metabolism and is targeted by drugs of the fibrate family to treated hypercholesteremia. Interestingly, in one of our studies a second nuclear receptor involved in the lipid metabolism (Liver X receptor; LXR [111]) was shown to transcriptionally modulated PDZK1. However, the activation of PPARα results in a higher HDL plasma level while the augmentation of PDZK1 expression lowers the plasma HDL cholesterol as it was described in different studies with knockout mice [110, 122, 123]. The scaffold protein influences the HDL cholesterol plasma level by its crucial role in stabilizing the HDL receptor (SR-B1) at membrane, that is essential for the uptake of the HDL particles[124, 125]. In the same study reporting LXR as regulator of PDZK1 we further showed that pregnane X receptor (PXR), a well-described regulator of drug metabolism and lipid homoeostasis[126-129], upon ligand-activation reduces the promoter activity and protein expression of the scaffold protein. Therefore, three nuclear receptors of the lipid homoeostasis are linked to PDZK1. However, dependent on the activated nuclear receptor the clinical outcome varies, as e.g. PXR induces the accumulation of triglycerides in the liver while no such effect was observed for LXR [130, 131]. In addition, PPARα elevated HDL plasma concentration whereas LXRα regulates several genes involved in the reverse cholesterol transport resulting in a reduced HDL plasma level [132]. Nevertheless, in which extend the PDZK1 regulation by these nuclear factors impacts the lipid homoeostasis *in vivo* remains to be clarified.

Unlike the direct interaction of LXR with PDZK1 we were not able to specify if the regulation by PXR is of direct or indirect nature. A possible hypothesis for the underlying mechanism, in case of an indirect regulation, is the involvement of the transcription factor hepatocyte nuclear factor 1 homeobox A (HNF1α). We reported HNF1α as a new regulator of PDZK1 and therefore of the "urate transportosome" in the kidney [133]. The presence of transcriptional factor HNF1α increased the mRNA as protein expression of the PDZK1 in renal proximal tubule epithelial cells (RPTEC). Furthermore, we showed a correlation of the mRNA expression of the homeobox with several transporters at apical and basolateral membrane of the proximal tubulus cells indicating HNF1α as a regulator of the "urate transportosome".

Taken together, our studies discovered new transcriptional regulators of PDZK1 that are involved in the thyroid homoeostasis, urate handling, lipid metabolism and pharmacokinetics of drugs (THR, HNF1α, LXR and PXR). In addition, we also described an interplay between the scaffold protein and the membrane transporter OATP2B1, a member of the SLCO family, which is also regulated by thyroid hormones. As indicated in the introduction the concept of "urate transportosomes" could possibly be applied for a lipid, a thyroid, or a drug "transportosomes" including PDZK1 (and to some extend OATP2B1). The evaluation of pharmacokinetics on the basis of transporters, receptors, and scaffold protein complexes seems to be a promising approach to a better understanding of ADME of xenobiotic and also endogenous substances. One may have the impression that this simplification to a functional unit would reduce the relevance of single proteins what would be a false conclusion. The research on each participant of a multiprotein complex will not be less relevant but rather the way to understand the inter-individual differences, which

will also have an influence on the “transportosomes”. Further studies on the topic of PDZK1, other scaffold proteins and the interaction partners will help to provide improved insight into the pharmacokinetics of drugs.

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Publikationsliste

Publications:

1. Prestin, K., J. Hussner, C. Ferreira, I. Seibert, V. Breitung, U. Zimmermann, and H. E. Meyer Zu Schwabedissen. „Regulation of Pdz Domain-Containing 1 (Pdzk1) Expression by Hepatocyte Nuclear Factor-1alpha (Hnf1alpha) in Human Kidney." Am J Physiol Renal Physiol 313, no. 4 (Oct 1 2017): F973-F83.

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4. Ferreira, C., P. Hagen, M. Stern, J. Hussner, U. Zimmermann, M. Grube, and H. E. Meyer Zu Schwabedissen. „ The scaffold protein PDZK1 modulates expression and function of the organic anion transporting polypeptide 2B1". Eur J Pharm Sci, 2018. 120: p. 181-190.

Manuscript:

5. Ferreira, C., R. Meyer, and H. E. Meyer Zu Schwabedissen. „ The nuclear receptors PXR and LXR are regulators of the scaffold protein PDZK1".